APOPTOSIS IN THE PROGRESSION OF IGA NEPHROPATHY

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Doctor of Philosophy

By

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ABSTRACT

This thesis examines the role of apoptosis or programmed cell death in the progression of IgA nephropathy, the commonest form of glomerulonephritis in the Western world.

The candidate first reviews the current literature on IgA nephropathy with particular focus on theories in the pathogenesis of disease. As the prognosis of the disease is so variable and at times difficult to predict, the candidate also attempts to summarise the literature regarding those clinical and histopathological features at presentation that are most significantly linked to disease progression. A distinction is made between primary and secondary IgA nephropathy as well as Henoch-Schönlein purpura, despite significant overlap between these disease entities.

Next, the candidate undertakes to summarise the current understanding of mechanisms involved in cellular apoptosis. The specific pathways that are involved in apoptosis are explored, in particular the Fas/Fas-ligand interaction to induce apoptosis, the Bcl-2 family of proteins and the caspase cascade. Finally, the candidate attempts to summarise the possible roles that apoptosis may play in the progression of chronic renal injury, particularly in IgA nephropathy. The role of apoptosis in the resolution of glomerular proliferation and the restoration of normal glomerular architecture is highlighted, as well as its possible role in the cell deletion that accompanies glomerulosclerosis. Of most importance the role apoptosis plays in the development of tubulointerstitial injury is examined. Possible mechanisms for the induction of tubulointerstitial cell apoptosis are explored with particular emphasis on the role proteinuria may play as a tubular epithelial cell toxin.

A database is constructed by the candidate of all patients presenting to The Alfred Hospital over a 25 year period with a diagnosis of primary IgA nephropathy. Clinical details at the time of renal biopsy are collected and renal biopsy samples are examined and scored for the degree of renal injury, both proliferative and sclerosing. The patients are then contacted to establish the course of their disease. Statistical analysis is then applied to the database to examine those features both clinical and histological that are most significantly linked to disease progression.

Renal biopsies from a subset of patients from the IgA nephropathy database are examined for the presence of apoptotic cells using the TUNEL technique applied to remnant renal biopsy tissues with normal nephrectomy samples as controls. The presence of apoptotic cells within glomeruli and the tubulointerstitium is noted and then correlated with the known patient clinical and histological parameters. The relationship between apoptotic cells and known renal prognosis is then examined by statistical analysis.

Remnant renal biopsy tissue from a subset of patients with IgA nephropathy is then examined for the presence of infiltrating leucocytes using immunohistochemistry. The intensity and subclass of infiltrating leucocytes is then correlated with known clinical and histological parameters as well as with renal prognosis. Further immunohistochemical analysis of the specimens is then performed to measure the expression of alpha smooth muscle actin. This is also correlated with renal prognosis.

To further explore the role of apoptosis in the progression of IgA nephropathy, the same renal tissue is further examined immunohistochemically for the expression of some of the proteins known to regulate apoptosis, in particular Bcl-2, Bax, Bcl-X_L and Fas-ligand. The expression of these proteins is then correlated with patient clinical and histological parameters as well with renal prognosis.

To further quantify the expression of the regulators of apoptosis in IgA nephropathy renal biopsy specimens, mRNA extracted from biopsy specimens are analysed using PCR techniques for the expression of Bcl-2, Bax, Fas and Fas-ligand. Competitive PCR is utilised to measure the expression of Bcl-2 and Bax mRNA from the specimens and real-time PCR is utilised to measure the expression of Fas and Fas-ligand mRNA. Normal nephrectomy specimens are used as controls. The measured levels of the proteins of interest are then correlated with known clinical and histological parameters as well as with renal prognosis.

All the results are then summarised and a brief discussion regarding possible future directions for the candidate's research takes place.

DECLARATION

This thesis is the sole work of the author and the material contained herein has not been previously published or written by any other person, except where due reference has been made in the text. The work was performed by the candidate in the Department of Medicine, Monash University Medical School, Alfred Hospital, Melbourne, Australia for the express purpose of this thesis. No part thereof has been previously submitted or accepted for the award of any other degree or diploma at this or any other University.

I certify that the writing of this thesis, the results and conclusions are my own work.

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Bonollo M, Menahem SA, Glare E, Mitchell J, Thomson NM, Stein-Oakley AN. TNFα gene polymorphisms in the progression of IgA nephropathy. ANZSN 38th Annual Scientific Meeting 2001.

Menahem SA, Langham RG, Stein-Oakley AN, Thomson NM. Apoptosis and its role in the progression of IgA nephropathy. ASN/ISN World Congress of Nephrology 2001.

Menahem SA, Maguire JA, Denver R, Stein-Oakley AN, Thomson NM. Identification of apoptotic cells in progressive IgA nephropathy and associated expression of pro-apoptotic proteins. ANZSN 39th Annual Scientific Meeting 2003.

Langham RG, Menahem SA, Gow RM, Dowling JK, Gilbert RE, Thomson NM. Progressive decline in renal function in IgA nephropathy is associated with elevated

gene expression of PDGF-B and PDGF-receptor B. ANZSN 39th Annual Scientific Meeting 2003.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Renal failure arising from acute, crescentic glomerulonephritis may present quite dramatically with a rapid decline in renal function. Often it can respond just as dramatically to aggressive therapy if instituted early [1] thus instilling a great sense of urgency in the treating physician. Whilst challenging and often gratifying to the treating physician with a response to treatment rate of up to 85% [1] this is a rare cause of renal failure. In Australia for example, crescentic glomerulonephritis accounts for only 13% of biopsy proven glomerulonephritis [2].

Much more commonly, renal failure arises from the slow and often relentless decline in renal function that accompanies most forms of chronic glomerular disease including diabetic nephropathy. Often to the frustration of the caring physician, despite aggressive therapies and risk factor modification, renal function continues to decline and the patient progresses to end-stage renal failure. In Australia and New Zealand these forms of renal injury account for more than 50% of patients commencing dialysis each year [3].

Whilst progressive injury to the kidney can be induced by a variety of different mechanisms each with its own pathognomonic histological pattern, at a certain point in disease progression these patterns often converge on a common pathway. This pathway involves cellular deletion accompanied by scarring and fibrosis of the glomerulus and the interstitium in the form of increased mesangial matrix and interstitial collagen deposition. Once this process has begun there is usually an unremitting decline in renal function. Many studies have confirmed the link between these histological changes and the progression of renal failure [4, 5]. The sequence of events that initiates this pattern of renal injury remains poorly understood, but understanding these events is vital if therapeutic intervention is to be made possible.

As the dialysis population continues to expand and the supply of cadaveric kidneys for transplantation remains limited, the true burden of the progression of

chronic renal injury is becoming evident. In Australia and New Zealand, in the ten years between 1990 and 2000, the number of patients on dialysis increased from 3,499 to 7,685, an increase of approximately 8% per year [3, 6]. Over the same period of time the number of renal transplants performed increased from 543 in 1990 to 636 in 2000, or by only 1.5% per year. This is despite an increase in the usage of living donor grafts by over 10% per year [3, 6].

The prevalence of end-stage renal diseases also increases as the population ages. For example, in Australia, between 1990 and 2000 the median age of new dialysis patients increased from 54 years to 61.2 years. The percentage of patients starting dialysis who were 65 years or older increased from 24% to 42% [3, 6]. As older dialysis patients are often ineligible for transplantation due to co morbidities, the number of patients permanently on dialysis will continue to climb, as will the total cost of their care. In the USA for example, it is projected that the cost of treatment for patients with end stage renal failure will increase from U.S. $16.74 billion in 1998 to U.S. $39.35 billion in 2010 [7].

In light of the progressive rise in the number of patients with chronic renal failure and dialysis dependence, it has become vital to both scientists and physicians alike to identify those factors that contribute to the progression of chronic renal failure. Only once these factors have been identified and understood, both at a molecular and clinical level, can interventions be trialed that may slow or halt disease progression.

For example, several trials have already shown that by reducing proteinuria, angiotensin-converting-enzyme (ACE) inhibitors may protect against the progression of chronic renal failure [8, 9]. At a molecular level, the selective inhibitor of platelet-derived growth factor (PDGF) receptor tyrosine kinase - STI 571 has shown promising results in ameliorating experimental proliferative glomerulonephritis, with the authors suggesting a possible role in human disease [10]. Many other strategies have been demonstrated to similarly limit glomerular damage in experimental models of proliferative glomerulonephritis. These include agents that reduce the expression of cytokines such as transforming growth factor β_1 (TGF- β_1) [11] and tumour necrosis factor-α (TNF-α) [12], as well as agents that inhibit T-cell number [13] and T-cell function.[14].

Unfortunately, the current pace at which the mystery of progressive renal damage is being unravelled seems unable to keep up with the ever-increasing burden of the disease.

1.2 Primary IgA Nephropathy

1.2.1 Introduction

In the late 1960s, with the application of immunohistochemical staining techniques to renal biopsies, Berger and Hinglais [15] described a cohort of 25 patients with moderate proteinuria and microscopic haematuria, who on renal biopsy had significant IgA deposition in the "intercapillary" space of glomeruli. This was associated with a "focal glomerulonephritis". Half of the patients had macroscopic haematuria associated with a "sore throat". Most patients had normal renal function, even 12 years after the initial onset of symptoms. Three of the patients were hypertensive and it seems apparent that the authors thought the disease to be relatively benign.

It was only in the subsequent years that the significance of these observations would come to be appreciated. It is now evident that IgA nephropathy is the commonest cause of glomerulonephritis in the world and although initially thought to be a benign condition, it frequently progresses to end-stage renal failure [16]. For example, in an Australian study, IgA nephropathy accounted for 34.1% of renal biopsies performed over a two year period [2]. In 2000, IgA nephropathy accounted for one third of patients with biopsy proven glomerulonephritis commencing dialysis [3].

1.2.2 Incidence and Prevalence

The true incidence of IgA nephropathy is difficult to ascertain. The relative differences seen in disease occurrence between populations more likely reflects local thresholds for renal biopsy and policies towards screening for urinary abnormalities rather than true incidence differences. For example, in Singapore where all males undergo urinary screening on induction to national service, IgA nephropathy accounted for 66% of primary glomerulonephritis [17] compared with 34% in Australia, where no such policy exists [2]. Similarly, due to the fact that only males undergo urinary

screening in Singapore, the male to female ratio for patients with asymptomatic disease in their population, is of the order of 5:1 compared to a ratio of 1:1 for patients presenting with symptomatic disease [18].

As a result of such screening programs and lower thresholds for renal biopsy, Asian patients tend to have more benign disease at diagnosis compared to other population groups. For example in the large retrospective review performed by Schena [19], 80.5% of Asian patients with IgA nephropathy had normal renal function at diagnosis compared to 69% in Europe. Similarly, in the European population, 40% of patients presented with macroscopic haematuria compared with 11.5% in the Asian population. It is unlikely that these differences reflect a true variation in the incidence and nature of the disease between these two population groups but rather the different methods used for disease detection. Thus great care must be taken when attempting to characterize the overall incidence and presenting features of IgA nephropathy.

Despite these reservations, it is clear that IgA nephropathy remains the commonest form of primary glomerulonephritis in countries where renal biopsy as a diagnostic technique is readily available [19]. As a way of avoiding sampling bias to identify the true prevalence of IgA nephropathy, Sinniah [20] performed immunofluorescence on renal tissue obtained from two hundred consecutive autopsies performed over a three-year period. The patients had no known history of renal disease and all died from trauma or suicide. He identified mesangial IgA deposition in 8 patients (4%) with 7 out of the 8 patients having some abnormality present in mesangial cell number or mesangial matrix. Only one patient however, had significant glomerular changes. It is interesting to observe this high prevalence of IgA nephropathy in an unscreened population, of the order of 4%, with 1 in 200 people having significant glomerular changes. Whilst only a relatively small study group, the unselected nature of the group makes these observations quite unique.

1.2.3 Renal Biopsy Findings

Unlike other glomerulonephritidies, IgA nephropathy is defined by demonstrating the presence of the immunoglobulin within the mesangium rather than by any specific morphological changes and thus the findings on light microscopy can vary greatly. The changes on renal biopsy typically associated with IgA nephropathy include

focal or diffuse mesangial cell proliferation associated with variable degrees of increased mesangial matrix [21]. The increased cellularity seen within the glomerulus most likely represents a proliferation of endogenous mesangial cells rather than an infiltration of inflammatory cells unlike other forms of proliferative glomerulonephritis. Despite an increase in overall glomerular cellularity, Nolasco et al [22] were unable to demonstrate a significant difference in glomerular leucocyte expression between non-proliferative glomerulonephritis and IgA nephropathy. Significant increases in leucocyte expression were observed in other forms of proliferative glomerulonephritis. Other authors have made similar observations [23].

Kincaid-Smith et al [24] however, showed that patients who underwent renal biopsy within 30 days of an episode of macroscopic haematuria had significantly higher number of glomerular crescents demonstrated, with an accompanying leucocytic infiltration when compared to patients with stable, low-grade haematuria. The leucocytic infiltrate was characterised by polymorphs and macrophages. This demonstrates that in IgA nephropathy particularly, the timing of the renal biopsy may well influence the nature of glomerular pathology seen, thus making it difficult to interpret changes observed in isolation from clinical parameters.

As mesangial matrix expands there is accompanying cell deletion which gives rise to areas of segmental and/or global glomerulosclerosis. This increase in matrix proteins may result from increased matrix production or reduced matrix breakdown. The major collagen component in normal matrix is type IV collagen, particularly the α1 and α2 isoforms [25]. Other components include the glycoproteins laminin and fibronectin. Studies of mesangial matrix proteins in glomerulosclerosis associated with IgA nephropathy have revealed that regions of sclerosis are composed of increased amounts of normal matrix proteins as well as proteins not usually present within glomeruli such as type III collagen [26].

More recent work has described the presence of type VII collagen in sclerotic regions in IgA nephropathy as well as other glomerulonephritidies. [27]. This collagen is preferentially expressed in tissue repair, particularly of skin. Type VII collagen is a fibrillary protein thought to play a role in basement membrane stabilisation and mechanical anchoring after injury. The authors, Muda et al [27] have suggested that in

glomerular injury, mechanical stress on epithelial cells induces aberrant collagen synthesis.

Other forms of glomerular pathology are observed but less frequently. As has been mentioned crescents may be present, especially in association with recent macroscopic haematuria [24]. A small group of patients present with florid crescentic glomerulonephritis associated with a rapid progression to end-stage renal failure [28]. Segmental necrotizing lesions, similar to those seen in vasculitis were observed in 10% of patients in a recent retrospective analysis [29]. The authors have suggested that this subgroup of patients may benefit from more aggressive therapies, such as those used in the treatment of anti-neutrophil cytoplasmic antibody (ANCA) positive vasculitis. A proportion of patients with IgA nephropathy, probably of the order of 10% have normal glomeruli on light microscopy.

The tubulointerstitial changes seen in IgA nephropathy are no different from those observed in other forms of chronic glomerulonephritis, apart from the presence of tubular red cell casts, which usually correlate with clinical haematuria [28]. Other changes not unique to IgA nephropathy include tubular atrophy, interstitial fibrosis and an interstitial infiltrate. The infiltrate is comprised predominantly of macrophages and T-cells, in particular $CD4^+$ T-cells [30]. It has long been recognized that it is the degree of interstitial injury that correlates closest with overall renal prognosis rather than the degree of glomerular injury [31]. Many studies in IgA nephropathy confirm this observation [5]. However in IgA nephropathy the degree of interstitial injury usually correlates quite closely with the degree of glomerulosclerosis.

As mentioned, the diagnosis of IgA nephropathy depends on the presence of immunoglobulin A being identified within the glomerulus. IgA is the only immunoglobulin identified in 25% cases. In 75% of cases there is also IgG or IgM or a combination of both present in association with the IgA [28]. Some studies have suggested that the extension of IgA deposition from mesangial areas to peripheral capillary loops may be associated with a worse outcome [32], but this has not been a universal finding.

1.2.4 Secondary IgA Nephropathy and Henoch-Schönlein Purpura.

For the purpose of this review it is important to distinguish between primary and secondary IgA nephropathy. As well as this, the disease entity Henoch-Schönlein purpura (HSP) must also be distinguished from primary IgA nephropathy despite several similarities between the two diseases.

There are several conditions other than primary IgA nephropathy that are associated with the deposition of immunoglobulin A within the glomerular mesangium. The commonest of these is chronic liver disease (CLD), in particular cirrhosis associated with alcohol excess [33]. Axelson et al [34] performed renal biopsies on a group of patients with liver cirrhosis at the time of liver transplantation. In this group with severe liver dysfunction, glomerular IgA deposition was observed in 11 out of 23 patients, with 2 of the patients showing other mesangial changes consistent with IgA nephropathy. Both patients with secondary IgA nephropathy had normal renal function at the time of transplantation and only one patient had low-grade proteinuria. Urinary sediment was not reported. There were no observed renal abnormalities at follow-up in any of these patients. Unfortunately the length of follow-up was relatively short and liver transplantation may have altered the natural course of the renal disease.

The clinical findings in patients with IgA nephropathy secondary to CLD are quite variable. In one series, up to 25% of patients with biopsy-proven secondary IgA nephropathy had significant urinary abnormalities, as defined by proteinuria >3g/24hrs or >5 red blood cells per high powered field [35]. This was associated with normal renal function or only mild renal impairment. Whilst the clinical findings at the time of diagnosis in this group of patients have been well described [36], the natural history of the renal lesion has not been well documented. It is interesting to note that many of the descriptive studies in this area arise from autopsy findings suggesting that the natural history of the renal lesion is overshadowed by the progression of the CLD.

Clues to the pathogenesis of IgA nephropathy associated with CLD are suggested by the observation that patients with CLD are often found to have elevated serum levels of immunoglobulin A [37]. In both Axelson's [34] and Newell's [36]

patient groups for example, elevated serum levels of IgA was almost a universal finding in patients with biopsy proven IgA nephropathy secondary to CLD.

Kutteh et al [37] demonstrated that patients with cirrhosis had significantly higher serum levels of polymeric IgA compared to normal patients. Polymeric IgA is derived from mucosal cells and usually comprises only a small proportion of serum IgA. In further studies, Kutteh et al [37] demonstrated that the liver is responsible in normal individuals for the secretion of polymeric IgA into hepatic bile. It has thus been postulated that with liver dysfunction, hepatic excretion of polymeric IgA is impaired, leading to higher serum levels of polymeric IgA. This may predispose individuals to the formation of IgA immune complexes which are selectively deposited in the mesangium of glomeruli [33]. There are certainly some similarities between the suggested pathogenesis of IgA nephropathy related to CLD and primary IgA nephropathy. This shall be discussed further in later sections.

Other diseases reported to be associated with mesangial IgA deposition are listed in Table 1.1 (from [28]).

I. Mucosal Diseases	
A. Chronic inflammation	Hypersensitivity (Sjögren's syndrome)
	Idiopathic (inflammatory bowel disease)
	Infectious (yersinosis, brucellosis)
B. Neoplasia	Bronchogenic and colon carcinoma
II. Hematological disease	Lymphoproliferative disorders
	Myeloproliferative disorders
III. Systemic Autoimmune	Ankylosing spondylitis
	Dermatitis herpetiformis

Table 1.1 Diseases associated with secondary IgA nephropathy.

Unlike primary IgA nephropathy, the natural history of IgA renal deposition associated with all of these conditions has been poorly explored. From what has been observed, it is unlikely that the renal course of secondary IgA nephropathy would mirror that of primary IgA nephropathy and thus it would seem sensible to exclude these patients from any discussion relating to primary IgA nephropathy. Furthermore, the progression of the underlying disease is more likely to dictate the prognosis of the patient rather than the associated renal lesion. This is certainly the case in advanced chronic liver disease, for example.

Whilst most authors agree that there is a causative relationship between mesangial IgA deposition and chronic liver disease, Bene et al [38] in their prospective necropsy study, observed that the incidence of IgA mesangial deposition in patients with alcoholic liver diseases was not significantly different from that seen in normal patients. Based on this observation they suggested that the relationship between chronic liver disease and IgA nephropathy was merely an overlap of two common diseases. Whilst most authors would disagree with this conclusion especially with regard to CLD associated IgA nephropathy, this may well be the case in some of the other conditions thought to be associated with mesangial IgA deposition.

Well before the advent of renal biopsy and immunofluorescence, Schönlein [39] and then Henoch [40] described a disease of young children characterized by rash, arthralgia, abdominal pain and macroscopic haematuria. The disease has since become known as Henoch-Schönlein purpura (HSP). HSP was linked to primary IgA nephropathy in 1968, with the demonstration of mesangial IgA deposits [41] on renal biopsies similar to those found in primary IgA nephropathy. Although the renal lesion of HSP is similar to that seen in primary IgA nephropathy, the natural history of the disease can be quite different. Davin et al have summarised some of these similarities and differences in their recent review [42]. Unlike primary IgA nephropathy, HSP is a systemic disorder affecting predominantly children under the age of fifteen. Arthralgia, abdominal pain, rash and glomerulonephritis are the hallmarks of the disease, with the rash characterised by a leukocytoclastic vasculitis associated with IgA deposition. On light microscopy the renal lesions in both diseases are quite similar in some respects. However in HSP, fibrinoid necrosis and crescent formation resembling the lesions seen in systemic vasculitis is a more common feature.

The renal presentations of the two diseases overlap considerably with haematuria and low grade proteinuria being the commonest renal presentation for HSP in one series [43], accounting for 57% of patients. In this same series 20% of patients at follow-up developed significant renal insufficiency or required dialysis, a similar number to that observed in primary IgA nephropathy. As in primary IgA nephropathy, those patients with more severe clinical presentations and more advanced histological lesions on biopsy were more likely to have progressive disease.

Whilst the pathogenesis of HSP is not known, several abnormalities in immunoglobulin A pathophysiology have been observed. Once again, these abnormalities show considerable overlap with those observed in primary IgA nephropathy. Circulating pathogenic IgA containing complexes (IgA-CC) have been observed in both diseases and are thought to bind selectively to glomerular mesangial cells [42]. For example, Allen et al [44] demonstrated an abnormality in IgA1 O-glycosylation particular to patients with IgA nephropathy and HSP. This abnormality was not seen in patients with HSP without renal involvement. It was suggested that this abnormality may compromise the hepatic clearance of IgA from the circulation and/or may favour the mesangial deposition of these molecules.

A disparity in the size and nature of other IgA-CC may explain the different systemic manifestations observed in the two diseases. However, it is likely that mesangial IgA-CC deposition triggers activation of similar pathways within the glomerulus, resulting in the significant overlap in the renal manifestations and prognosis of both diseases.

1.2.5 Presenting Features of IgA Nephropathy

Guiseppe D'Amico, in the introduction to his recent review of the natural history of idiopathic IgA nephropathy [5] highlights the problem of describing the clinical manifestations of the disease. Whilst one patient may present with recurrent episodes of syntharyngitic haematuria with no apparent affect on renal function even after many years, another may present at a young age with severe hypertension and advanced renal failure with no previous signs of illness. Other presentations may include the nephrotic syndrome, nephritic syndrome and that of rapidly progressive glomerulonephritis. It was this variability and often unpredictability in presentation that led D'Amico in his

conclusions to suggest that IgA nephropathy is not a single disease but rather "a spectrum of diseases sharing a common pathogenic marker". This sentiment is also shared by other authors [45]. For this reason it is often difficult, even precarious to make to many sweeping generalisations about IgA nephropathy.

Despite this, due to the multitude of papers written to describe large cohorts of patients with IgA nephropathy, certain clinical patterns do emerge. In D'Amico's review [5] he draws his conclusions on the natural history of IgA nephropathy from only 30 of these studies. The reviewer drew upon these selected studies only as he felt that they were the most stringently designed publications based on his own strict criteria. These criteria included large patient numbers, prolonged follow-up and accurate statistical evaluation. From his work it would seem reasonable to suggest that in primary IgA nephropathy the average age of presentation is between 25 and 35 years of age, approximately one third of patients have renal impairment at diagnosis, the majority of patients have low grade proteinuria with approximately 10% having nephrotic range proteinuria and approximately 40% of patients give a history of macroscopic haematuria. He suggests that the actuarial renal survival at 10 years is somewhere between 80 and 85% and that the disease is more common in males than females with a ratio in the order of 3:1.

In their original description of IgA nephropathy, Berger and Hinglais [15] noted that at least half of their patients had episodes of macroscopic haematuria at presentation, usually in association with an upper respiratory tract infection. In light of this and the fact that it was incorrectly assumed that the disease was innocuous, the condition was originally known as benign recurrent haematuria. Since the original description of the disease, macroscopic haematuria has been described in association with a wide variety of usually mucosal infections, as well as in association with vigorous exercise.

In countries where urinary screening programs are linked with a pro-active renal biopsy policy, macroscopic haematuria is a less prominent feature of the disease. For example in a series from Singapore [17], where urinary screening of especially males is routine, only 20% of patients with biopsy proven IgA nephropathy had a history of macroscopic haematuria. Sixty-five percent of patients had asymptomatic disease detected via health screening.

Most investigators conclude that macroscopic haematuria is, at least by univariate analysis, associated with more benign disease. For example Haas [46], in his description of 244 patients with IgA nephropathy, found that those patients with gross haematuria had a better renal prognosis than those without.

It has been suggested that patients with macroscopic haematuria have more benign disease as a result of discontinuous exposure to the causative pathogen. This triggers exacerbations and remissions in disease. Patients without macroscopic haematuria may have continuous, low-grade exposure to the triggering pathogen resulting in more chronic, progressive renal damage. It is also possible that the symptom of macroscopic haematuria results in earlier diagnosis of disease and thus allows for more aggressive control of risk factors and delay of disease progression.

However many other studies, particularly those using multivariate analysis, have not identified macroscopic haematuria as being associated with a better disease prognosis, suggesting these theories may be only partly correct. As observed by Haas [46], when corrected for serum creatinine at the time of biopsy, macroscopic haematuria was no longer significantly associated with a better prognosis. Furthermore, it must be noted that in patients presenting with IgA nephropathy over the age of 40, macroscopic haematuria is rarely a feature of the disease. This suggests that at least in older patients other variables control disease progression.

A small proportion of patients with IgA nephropathy, probably of the order of 5%, present with a rapidly progressive, crescentic glomerulonephritis. An equally small number of patients with IgA nephropathy present with the nephrotic syndrome manifest by heavy proteinuria, hypoalbuminaemia and peripheral oedema. It is not clear whether both these groups of patients have primary IgA nephropathy or an "overlap" syndrome that involves two distinct glomerulopathies. As already observed, IgA deposition can be present within glomeruli without any clear pathological effect and thus IgA deposition in these patients may be a secondary phenomenon.

The association between IgA nephropathy and hypertension requires some discussion. Hypertension is a common feature of most forms of chronic glomerular disease. It is often multifactorial and secondary to the effects of renal damage regardless of the underlying pathology. As a presenting feature of IgA nephropathy, it is observed in approximately 10-20% of patients. For example Alamartine et al [47], in their series

of 282 patients with IgA nephropathy, observed a degree of hypertension as defined by a diastolic blood pressure of greater than 95mmHg, in 9% of patients. Yagame et al [48], in their cohort of patients, correlated the degree of hypertension with the severity of the renal lesion observed at biopsy. This is in keeping with the idea that hypertension observed in IgA nephropathy is secondary to the degree of renal damage at the time of biopsy. Furthermore, it is rare for hypertension per se to be demonstrated as an independent predictor of progressive renal disease on multivariate analysis.

However, there is a significant incidence of severe or malignant hypertension in association with IgA nephropathy. This is out of keeping with the expected incidence of malignant hypertension for the degree of renal damage observed. Unfortunately, many descriptive studies do not differentiate between mild and severe hypertension, making this a poorly explored aspect of IgA nephropathy. In those studies that do differentiate between degrees of hypertension, the incidence of severe or malignant hypertension can be very high. In a population of patients with IgA nephropathy from India for example, Muthukumar et al [49] observed accelerated or severe hypertension in 21% of patients. It should be noted that the patient population in this study had quite severe disease, with 60% of patients having renal impairment at presentation. Most studies observe an incidence of between 5 and 15% of malignant hypertension.

Malignant hypertension is quite commonly associated with a more rapid decline in renal function as observed by Subias et al [50]. In this patient population from Spain, 15% of individuals with IgA nephropathy had malignant hypertension as a presenting feature. This group had a much more rapid decline in renal function compared to a similar group with only mild to moderate hypertension. Patients with malignant hypertension in this study group did have significantly worse renal function at presentation. As multivariate analysis of the data was not performed it is unclear whether the hypertension was an independent predictor of worse renal outcome or just a reflection of poor renal function. Biopsies from this patient group did however reveal more severe glomerulonephritis with a higher percentage of crescents and segmental sclerosis, suggesting perhaps a more aggressive pattern of glomerular injury in this patient population.

1.2.6 Pathogenesis of IgA Nephropathy

The underlying cause of primary IgA nephropathy remains poorly understood. Furthermore, in light of what has already been discussed, it would be unwise to assume that all patients with IgA nephropathy share a single, common pathogenesis. The observed association between mucosal inflammation, macroscopic haematuria and mesangial IgA deposition suggests an abnormality in the production, catabolism or excretion of immunoglobulin A. For the purpose of this review it is convenient to separate these three processes. However, it may well be that a combination of abnormalities in the processing of immunoglobulin A gives rise to IgA nephropathy.

There is strong clinical evidence that IgA nephropathy is a systemic disorder particular to a given individual. Koselj et al [51] described seven year follow-up of three, living related kidney donors with silent IgA nephropathy and their corresponding recipients. It is revealing to note that once the kidneys were transplanted to the recipients, subsequent renal biopsy revealed complete or almost complete resolution of mesangial IgA deposition. The long-term function of these kidneys, despite the earlier diagnosis of silent IgA nephropathy was excellent. It must be noted that one of the donors with silent IgA nephropathy, after nephrectomy went on to develop overt IgA nephropathy with associated renal failure.

1.2.6.1 Immunoglobulin A Production

Whilst it is tempting to implicate excess or abnormal mucosal IgA production in the pathogenesis of IgA nephropathy, most studies implicate the bone marrow as the source of mesangial deposited IgA.

IgA is produced in two isotype subclasses; IgA_1 and IgA_2 by plasma cells within the bone marrow and lining mucosal surfaces. Unlike bone-marrow derived IgA, mucosal IgA rarely enters the systemic circulation. Mucosal IgA is of both subclasses however bone marrow derived IgA is exclusively IgA_1. To determine whether mesangial IgA seen in IgA nephropathy is of subclass IgA_1 or IgA_2, Lomax-Smith et al [52] examined renal biopsies from patients with primary IgA nephropathy as well as HSP and chronic liver disease associated IgA nephropathy. Staining biopsies for IgA_1 or IgA_2 subclasses, they demonstrated that for the most part mesangial IgA deposits in these conditions were IgA_1 in origin. Of note 4 of 39 patients demonstrated no IgA_1

staining and 20% of patients also had IgA_2 staining, again demonstrating the difficulties in identifying a *single* pathogenesis for the disease.

The bone marrow is thought to be the origin of the IgA_1 in IgA nephropathy. Harper et al [53] examined bone marrow biopsy specimens from seven patients with IgA nephropathy compared to seven matched controls. They noted a significant increase in IgA producing cells within the bone marrow of patients with IgA nephropathy. This corresponded to an increase in serum IgA for these patients. Unfortunately it is clear that increased production of IgA alone does not trigger the onset of disease. This is demonstrated by patients with extremely high serum IgA levels as a result of multiple myeloma who have not been shown to develop glomerulonephritis.

Therefore, rather than simply an increase in total circulating IgA, others have tried to identify an abnormality in the IgA molecule itself that predisposes it to mesangial deposition in these patients. As has already been discussed, Allen et al [44] described abnormal O-glycosylation of immunoglobulin A in patients with IgA nephropathy and HSP. IgA_1 was eluted from glomeruli from patients with IgA nephropathy. As well as this, circulating IgA from these patients and control subjects was isolated. It was demonstrated that the IgA bound to glomeruli in patients with IgA nephropathy differed fundamentally from circulating IgA in the same patients and normal controls. The glomerular IgA had significantly higher lectin binding compared to circulating IgA suggesting an increased frequency in truncated O-glycan chains. These truncated chains suggest a lack in terminal galactosylation and sialylation of N-acetyl galactosamine present within the hinge region of immunoglobulin A. The authors suggest that this abnormality in the IgA molecule may predispose it to mesangial cell binding.

The same authors have identified a deficiency in leucocyte β1,3-galactosyl transferase activity in patients with IgA nephropathy, the enzyme responsible for galactosylation of the O-glycan chains [54]. This enzymatic deficiency may give rise to the abnormally glycosylated IgA. It is postulated by the authors that this abnormal IgA molecule has increased affinity for mesangial cells.

1.2.6.2 Immunoglobulin A Catabolism

The link already described between chronic liver disease and IgA mesangial deposition suggests that impaired hepatic catabolism of immunoglobulin A may play a role in primary IgA nephropathy. As already mentioned, Kutteh at al [37] investigated the role the liver plays in the excretion of IgA by analysing the contents of bile secretions from patients with T tube drainage of their common bile ducts. They observed that in normal patients (i.e. patients without liver cirrhosis) there were significant amounts of IgA in biliary secretions in both monomeric and polymeric forms. The amount of polymeric IgA present within bile far exceeded that present in serum, suggesting an active transport mechanism for polymeric IgA from the hepatic circulation to bile. Whilst damage to hepatocytes may restrict this active transport process giving rise to secondary IgA nephropathy, it is not clear whether an abnormality in IgA excretion contributes to primary IgA nephropathy.

Roccatello et al [55] demonstrated prolonged circulation of IgA_1 immune complexes in some patients with known primary IgA nephropathy as well as in patients with chronic liver disease. In patients with primary IgA nephropathy the liver was demonstrated to be the main site for removal of IgA_1-IgG immune complexes with some contribution from the spleen. Patients with chronic liver disease demonstrated slower liver clearance of IgA_1-IgG immune complexes with a greater contribution from the spleen. It was noted that there was a correlation between delayed liver clearance of IgA immune complexes and renal failure in those patients with primary IgA nephropathy.

Not all catabolism of IgA is performed by the liver. Grossetete et al [56] studied the IgA Fc receptors present on blood and tissue myeloid cells in patients with IgA nephropathy compared with controls. These receptors are thought to also play a role in clearance of IgA immune complexes by receptor mediated endocytosis. The investigators demonstrated enhanced binding of endogenous IgA to cells from patients with IgA nephropathy compared to controls. The receptor molecule was shown to be of a higher molecular weight in patients with disease compared to those of controls. The authors suggested that the larger, higher affinity IgA Fc receptor demonstrated in patients with IgA nephropathy may cause prolonged binding of IgA to the receptor. This

in turn may lead to down regulation of receptor expression and therefore increased levels of circulating immune complexes already demonstrated in these patients.

1.2.6.3 Immunoglobulin A Mesangial Deposition

It is clear that whether the abnormality lies within the production of IgA or the catabolism of IgA, it is the deposition of IgA within the glomerular mesangium and the inflammatory response this elicits that is the key initiator of the disease process. It must be remembered however, that some patients demonstrate IgA deposition within their renal mesangium with perfectly normal renal parameters and no glomerular pathology.

Only recently Barratt et al [57] discovered a novel mesangial cell receptor that recognises the Fc portion of IgA. This receptor was demonstrated to bind all isoforms of IgA but with particular affinity for polymeric IgA. The receptor was shown to be distinct from the Fc receptor already demonstrated on myeloid cells although the two receptors shared some homology.

Several *in vitro* studies have examined the effect binding IgA has on mesangial cells. Amore et al [58] for example, demonstrated that abnormally glycosylated IgA isoforms isolated from patients with IgA nephropathy, significantly depressed proliferation of cultured mesangial cells and increased apoptosis rates. Westerhuis et al [59] on the other hand, demonstrated that incubation of human mesangial cells with human IgA lead to a significant increase in interleukin-6 production, a potent cytokine linked to mesangial cell proliferation and increased synthesis of extracellular mesangial matrix. These results are more in keeping with the initial renal response observed in human IgA nephropathy.

However it is clear from what has already been observed, that IgA deposition within the mesangium does not induce the same inflammatory response for all patients. There is no clear understanding to date of which factors in any one individual control the type of response elicited by IgA mesangial deposition. Possibly unidentified genetic factors may determine this.

The glomerular inflammatory response observed, following IgA mesangial deposition is likely to be mediated by cytokines such as interleukin 6 (IL-6), platelet-derived growth factor (PDGF) as well as by local complement activation. As mentioned, IL-6 has been demonstrated to play a key role in the stimulation of mesangial cell proliferation and the increased synthesis of mesangial matrix [60].

In keeping with this, Harada et al [61] have linked increased urinary excretion of IL-6 to disease progression in a cohort of patients with IgA nephropathy followed over 8 years. They noted that those patients with a 24-hour urinary IL-6 excretion rate of greater than 2.5ng/day at presentation, had a 7.8 fold increased risk of disease progression.

Another cytokine demonstrated to play a role in stimulating mesangial cell activation is PDGF. Several studies have described an increase in mesangial expression of PDGF in patients with IgA nephropathy [62]. Niemir et al [63] demonstrated a correlation between increased mRNA expression of PDGF and PDGF receptors and the degree of glomerular proliferation and fibrosing interstitial lesions in patients with IgA nephropathy compared to controls. Highlighting the role of PDGF in glomerular activation, Gilbert et al [10] have demonstrated the suppressive affect of the PDGF receptor tyrosine kinase inhibitor - STI 571 on glomerular proliferation in experimental models of IgA nephropathy.

However, in IgA nephropathy and most other forms of glomerulonephritis the cause of disease progression and renal failure is not glomerular proliferation but rather the development of glomerulosclerosis and tubulointerstitial atrophy. As the disease progresses a different group of pro-sclerotic cytokines are expressed that give rise to this characteristic lesion. Transforming growth factor β1 (TGF-β1), a potent fibrogenic cytokine is most likely to play a pivotal role in this aspect of disease progression. Yang et al [64] were able to isolate glomeruli by micro-dissection from patients with a variety of forms of glomerulonephritis and measure the expression of TGF-β1 mRNA. They were able to show a direct correlation between the degree of glomerulosclerosis observed and the glomerular expression of TGF-β1.

In vitro studies on the effect of TGF-β1 on cultured mesangial and tubular epithelial cells, such as work done by Gore-Hyer et al [65], demonstrate the fibrogenic nature of TGF- β1. Both cell lines were observed to produce increased amounts of collagen proteins when exposed to TGF-β1, as well when exposed to another pro-fibrotic cytokine, connective tissue growth factor (CTGF).

Goumenos et al [66] have linked heavy proteinuria, a strong predictor of disease progression, to the tubular expression of TGF-β1. They made several interesting observations in renal biopsies from a group of 25 patients with nephrotic range

proteinuria compared to normal subjects and to patients with mild IgA nephropathy. Firstly they demonstrated in patients with heavy proteinuria that TGF-β1 was expressed in the cytoplasm of tubular epithelial cells. Urinary TGF-β1 excretion was significantly elevated in these patients and was shown to diminish with the reduction of proteinuria following therapy. Glomerular expression of TGF-β1 was only seen in those patients with mesangial proliferation associated with IgA nephropathy.

1.2.7 Predictors of Disease Progression in IgA Nephropathy

Hundreds of papers have been written to describe and correlate the clinical and histological parameters of IgA nephropathy with disease outcome. Most of them attempt to identify those features at presentation that are associated most strongly with disease progression. As is clear from the discussion so far, only some patients with IgA nephropathy suffer from a progressive decline in renal function whilst many others follow a relatively benign course. The challenge is to identify those patients at presentation who will follow a more progressive disease course and thus target this patient group for specific treatments as well as aggressive risk factor modification.

Caution must be applied when interpreting many of the papers written describing patient populations with IgA nephropathy. As is clear from earlier discussions it is unlikely that any two patient populations are alike. Patients' racial origins, genetic history, physicians' thresholds for renal biopsy, physicians' approaches to treatment, screening policies for populations and methods of biopsy interpretation are just some of the features that will vary greatly from population to population.

Despite these limitations, as mentioned D'Amico [5] performed a meta-analysis of all published patient population groups in an attempt to best summarize the current literature on the natural history of IgA nephropathy. He applied very strict criteria in order to select the 30 papers he would include in this meta-analysis.

D'Amico's conclusions as to the clinical and histological factors that best predict disease outcome in primary IgA nephropathy are summarised in Table 1.2 (reproduced from [5]). He divided the predictors of disease progression into strong, intermediate and weak categories based on the strength of the evidence drawn from the meta-analysis.

Strength of Evidence	Predictor of Disease Progression
A. Strong Predictors	Elevated serum creatinine at presentation
	Severity of proteinuria at presentation
	Widespread global glomerulosclerosis
	Marked tubulointerstitial lesions
B. Intermediate Predictors	Arterial hypertension at presentation
	Marked extracapillary proliferation
C. Weak Predictors	Older age at presentation
	Male sex
	Absence of recurrent macroscopic haematuria
	Diffuse mesangial hypercellularity
	Marked arterial hyalinosis
	Extension of IgA deposits into capillary loops

Table 1.2 Clinical and histological predictors of disease progression in IgA nephropathy. (From [5])

As is clear from the meta-analysis, the strongest predictors of disease progression in IgA nephropathy are the degree of renal impairment at presentation, the degree of proteinuria at presentation and the degree of glomerular and interstitial fibrosis on renal biopsy. None of these features are particularly unique to IgA nephropathy as the progression of most forms of glomerulonephritis is linked to these factors. It is surprising to note that prognostic features traditionally thought to be unique to IgA nephropathy, such as male sex and the absence of macroscopic haematuria are only weakly linked to disease progression on meta-analysis.

Overall D'Amico suggested that on average an untreated patient with IgA nephropathy has a 10 year actuarial renal survival rate of between 80% and 85%. Caution must be utilised when applying such statistics to individual patients. As has already been described, the disease can affect individuals in an idiosyncratic manner. One should conclude that in a large cohort of patients with primary IgA nephropathy approximately 20% will have progressive disease. It is difficult to control for the large number of variables that this figure is dependant upon.

The concept of "a point of no return" for IgA nephropathy was introduced by D'Amico in earlier descriptive paper. In 1993 D'Amico et al [67], based on a small cohort of patients with IgA nephropathy, suggested that once an individual's serum creatinine reached 3mg/dL (0.265 mmol/L) it was inevitable that their renal function would continue to decline, approaching end-stage renal failure. Schöll et al [68], examining a much larger group of patients with IgA nephropathy, confirmed the existence of this "point of no return" at a serum creatinine of 3mg/dL (0.265 mmol/L). Schöll et al observed that once this level of renal impairment was reached by patients, no remissions were observed and renal function continued to decline in all patients.

The likelihood is that this degree of renal impairment corresponds to a critical degree of glomerulosclerosis and tubulointerstitial fibrosis, such that progression of disease is relentless. This phenomenon is certainly not unique to patients with IgA nephropathy, but does give some insight into the pathogenesis of progressive renal disease.

Despite all these observations regarding disease progression, a recent paper by Szeto et al [69] have highlighted the difficulties encountered in trying to predict the course of disease in a patient population. Szeto et al [69] described a cohort of 72

patients with primary IgA nephropathy detected by screening urinalysis. This cohort of patients had very mild disease with minimal proteinuria and well preserved renal function at the time of biopsy. It was anticipated, from what has already been observed, that this group of patients over time would follow a relatively benign course. After 7 years follow-up however, the authors noted that a third of patients had developed proteinuria of greater than 1g/day, one quarter of patients had become hypertensive and 7% of patients had developed impaired renal function.

It is possible that early identification of patients with IgA nephropathy using screening urinalysis will detect a larger group of patients with relatively benign features at diagnosis but progressive disease at follow-up. Alternatively, the patient population examined by Szeto et al may have more aggressive disease due to as yet unidentified genetic factors. This may be especially true in an Asian population compared to other patient groups.

1.2.8 Treatment of IgA Nephropathy

A variety of treatment options for IgA nephropathy have been examined but none have been proven to consistently and reliably alter the course of the disease. The nature of the disease with its variable forms of presentation and at times unpredictable prognosis can make it difficult to apply standardised treatments to large groups of patients. Furthermore, the slow progression of the disease in some individuals may necessitate prolonged treatments with potentially toxic therapies, a course that may be difficult to justify in some patient groups.

Treatment options can be divided into two categories; general measures including risk factor modification and specific targeted treatments. General measures include weight loss, smoking cessation, reduction in alcohol intake, control of hypertension and treatment of hyperlipidaemia. Targeted treatments include fish oil therapy, corticosteroids and more aggressive cytotoxic therapy.

Although intuitively risk factor modification should reduce the risk of disease progression in IgA nephropathy, there are very few trials that actually demonstrate this. Apart from reliable evidence on the benefits of anti-hypertensive therapy on disease progression, there is mostly retrospective data to support the benefits of other risk factor modification.

For example, Orth et al [70] identified cigarette smoking as a risk factor for disease progression in a large cohort of patients with primary IgA nephropathy. Although it has not clearly been demonstrated, it stands to reason that smoking cessation would delay disease progression in these patients.

Hypertension has been demonstrated to be a reasonably strong predictor of disease progression in IgA nephropathy as well as in most forms of renal disease. Many trials have demonstrated the benefits of anti-hypertensive therapy in slowing the progression of renal disease. Angiotensin converting enzyme inhibitors (ACEI) have in particular been demonstrated to slow the progression of many forms of renal disease, largely as a result of their anti-proteinuric affect. For example, Rekola et al [71] demonstrated the benefit of ACEI in delaying disease progression in a group patients with primary IgA nephropathy. Enalapril was shown to significantly slow the progression of renal disease compared to beta-blocking agents for the same level of blood pressure control. In a larger trial that included patients with other forms of glomerulonephritis, the GISEN group [8] demonstrated the benefit of the ACEI ramipril in reducing proteinuria along with a parallel delay in the progression of renal disease.

With the advent of the newer angiotensin-II receptor antagonists (A-IIRA), more recent trials have demonstrated the benefits of these agents in delaying renal disease progression particularly in patients with diabetic nephropathy [72]. Showing some promise, a recent trial examining the effect of combining ACEI and A-IIRA in patients with non-diabetic renal disease demonstrated a significant reduction in disease progression in those patients on combined treatment [73]. Once again the benefits were thought to be due to the added anti-proteinuric affect of the two agents when combined.

The benefits of more specific therapies in delaying disease progression in IgA nephropathy remain more controversial. Whilst there have been many publications examining the benefits of various treatment options in IgA nephropathy, few of them can be described as acceptably randomised and controlled. In the most recent meta-analysis on the subject, Strippoli et al [74] selected only 10 trials from a possible 1196 for analysis. The benefits of treatment in this analysis were divided into those treatments for IgA nephropathy that have been shown to delay disease progression and those that have been shown to reduce proteinuria.

In summary, Strippoli and his colleagues concluded that corticosteroid treatment for IgA nephropathy probably has a benefit in reducing proteinuria but has not been shown to consistently retard disease progression. Cytotoxic therapy including the use of drugs such as cyclophosphamide and azathioprine has some benefit in delaying disease progression and reducing proteinuria. Fish oils probably have no benefit on either parameter. Again it should be noted that in the ten trials examined no two study populations were exactly alike. Patient populations differed on the basis of average age, the degree of renal failure at the commencement of treatment and the degree of proteinuria. This factor further limited the authors' ability to draw definite conclusions from their meta-analysis.

It would therefore be reasonable to suggest that current treatment of IgA nephropathy would include lifestyle risk-factor modification, aggressive treatment of hypertension preferably with an ACEI or A-IIRA or a combination of the two agents for patients with at least moderate proteinuria. Consideration should be given to corticosteroid treatment in patients with ongoing heavy proteinuria and well preserved renal function. Cytotoxic therapy may be considered in patients with a progressive decline in renal function. However the benefits of these agents must be weighed up against their toxicities, in particular opportunistic infection and malignancies.

1.3 Apoptosis and its Regulation

1.3.1 Introduction

The term "apoptosis" was coined by Kerr et al in 1972 [75] to describe a process of cell death not linked to inflammation and cellular necrosis. It was observed by the authors to occur in a number of cell lines and tissue types with distinct morphological characteristics. Whilst acute inflammation was well established as a cause of cellular injury and organ failure, the process of quiescent or programmed cell death as a vital part of tissue development as well as tissue injury and organ failure became a new area for investigation. This newer focus was even further intensified some 15 years later with the realisation that genes involved in the regulation of cell death may play a role in the pathogenesis of some cancers [76].

The role of apoptosis in tissue kinetics was summarised by Hengartner in his recent review of the biochemistry of apoptosis [77]; "As important as cell division and cell migration, regulated (or programmed) cell death allows the organism to tightly control cell numbers and tissue size, and to protect itself from rogue cells that threaten homeostasis".

The uniqueness of the process of apoptosis is highlighted by the morphological changes apparent in a cell that is undergoing programmed cell death. This series of changes, as summarised by Wyllie [78], begins with the cell separating from its neighbours with a loss of specialised membrane structures such as microvilli and desmosomes. The cell membrane is then observed to undergo "blebbing", separation of small parcels of cytosol, usually devoid of organelles. Following this there is rapid and irreversible condensation of cytoplasm and nuclear chromatin. Accompanying this condensation of nuclear chromatin, there is cleavage of chromatin to yield oligonucleosomal DNA fragments. It is this fragmentation of DNA that forms the basis of many of the diagnostic tests utilized to identify cells undergoing apoptosis.

Soon after the condensation of cytoplasmic and nuclear chromatin the cell divides into a cluster of membrane bound bodies each containing a variety of organelles. These smaller cell components are very quickly identified by surrounding phagocytes and are digested and removed from the tissue without the incitement of any inflammatory response. The process is thought to be so efficient that apoptotic cells are assumed to be visible by light microscopy for only a matter of hours.

The stimuli that induce these morphological changes within a given cell are quite varied. They range from a variety of physiological stimuli such as local cytokine production, noxious stimuli such as ultraviolet (UV) radiation as well as the withdrawal of cellular nutrients and growth factors. Cytokines, such as those in the tumour necrosis factor family of proteins, have been demonstrated to bind to cell membrane receptors to play a key role in the activation of cellular apoptotic cell death [79]. Cotton et al [80] demonstrated a dose dependant response to UV radiation in the *in vitro* induction of apoptosis in human keratinocytes. Withdrawal of growth factors such as interleukin-2 has been shown to have a pro-apoptotic effect on cultured T-lymphocytes [81].

These varied stimuli for apoptosis, via a myriad of intracellular pathways, converge upon a single common group of enzymes to trigger the initiation of the cycle

that leads to inevitable cell death. This group of enzymes are known as caspases, named for the ubiquitous presence within these enzymes of Cysteine-containing proteASES that show a predilection for cutting ASPartic acid residues (hence the term CASPASE). Once this group of enzymes are activated, the morphological changes of apoptosis mentioned earlier become evident within the cell. Regardless of the initial stimulus, it is the caspase group of enzymes that ultimately induce apoptosis, prompting Hengartner to label them as "the central executioners of the apoptotic pathway" [77].

Given that caspases have been observed to be present in all mammalian cells as well as in most multicellular organisms, it stands to reason that in normal cell life there must be an active process of caspase inhibition that ensures cell survival. Only with removal of this inhibitory influence can caspase activation take place, leading to cellular apoptosis. The Bcl-2 family of proteins have been shown to play this crucial role in the inhibition and activation of the capases. In mammals, this large family of proteins contains both pro-apoptotic and anti-apoptotic members. Via complex interactions between various members of the Bcl-2 family involving the formation of heterodimers, the balance between pro-apoptotic and anti-apoptotic proteins determine the fate of a cell at any given time. If the balance favours apoptosis, then via specific mitochondrial pathways involving the electron carrier cytochrome *c*, the caspase group of enzymes are activated.

Given the complexity and expansive nature of these pathways that induce cellular apoptosis, the candidate has chosen to focus on those specific aspects of apoptosis that are potentially involved in glomerular and tubulointerstitial injury.

1.3.2 Fas and Fas Ligand as Potent Inducers of Apoptosis

Early in the process of deciphering pathways to apoptosis, Trauth et al [82] identified a receptor on the surface of human lymphocytes, APO-1, that when activated by monoclonal anti-APO-1 antibody induced cellular apoptosis. Further *in vivo* studies demonstrated the affect of the antibody on human B cell tumours transplanted into mice. A single intravenous injection of the anti-APO-1 antibody into the mice induced tumour regression within days, once again mainly through the induction of apoptosis of tumour cells.

At the same time Yonehara et al [83] demonstrated that the binding of monoclonal antibody to the APO-1 receptor or Fas as they termed it, induced cellular changes indistinguishable from those induced by the cellular binding of the cytokine tumour necrosis factor (TNF). The Fas antigen was identified as distinct from the TNF receptor based on molecular weight analysis. Both studies pointed to a cell surface receptor similar but distinct from the receptor of TNF, that when activated was a potent inducer of cellular apoptosis.

As it became clear that target cells contained celluiar receptors that induced rapid programmed cell death, it was thought likely that effector cells such as T-lymphocytes would express a corresponding ligand. In 1993 Suda et al [84] identified Fas ligand as a type II transmembrane protein belonging to the TNF family. They demonstrated the ligand to be present on activated T-cells and once bound with Fas on receptor cells was able to induce apoptosis.

The Fas receptor has since been demonstrated to be expressed widely across cell types. It is a type I transmembrane receptor that contains a specific death domain within its cytoplasmic tail. Activation of the receptor by the binding of Fas–ligand for example, induces a complex series of intracellular protein rearrangements that leads to the transduction of the apoptotic signal. As summarised by Krammer recently [85], the process involves the formation of a complex of proteins around the cytoplasmic tail of the receptor, known as the death initiating signalling complex (DISC). Initially there is binding of the intracellular Fas-associated death domain protein (FADD) via its own death domain to the death domain of Fas. The FADD binds to a homologous region of a second protein, Fas-activated protein like interleukin converting enzyme (FLICE) also known as procaspase 8. FLICE is activated proteolytically and active caspase 8 is released into the cellular cytoplasm. With activation of caspase 8, other caspases are activated including caspase 3. These two caspases in particular are responsible for triggering the final enzymatic changes that lead to irreversible cell death.

Fas-ligand on the other hand is expressed in a much more limited capacity when compared to Fas. It appears on cytotoxic T lymphocytes and targets Fas-expressing cells to induce cell death [86]. In this way for example hepatitis C virus infected cells, which have increased cellular expression of Fas, can be targeted for deletion [87]. Expression of Fas ligand has also been reported on activated B cells and natural killer cells [88].

The Fas/Fas-ligand interaction to induce cell death has been demonstrated to play a role in immune regulation, embryogenesis and in the pathogenesis of disease. Whilst its role in the removal of defective or virally infected cells may be overshadowed by other cytotoxic immune mechanisms such as the perforin/granzyme B pathway, the Fas system may be the main mechanism for immune response down-regulation. As demonstrated by Ju et al for example [89], activated T cells are selectively deleted by up-regulation and then cross-linkage between Fas and Fas ligand. With cross-linkage of the receptor and its ligand, activated T-cells are deleted via apoptosis. This process may be central to the removal of activated T cells from the periphery following an immune response.

Studies of Fas/Fas ligand expression on hepatocytes have suggested a role for the Fas system in the induction of liver failure in alcoholic or hepatitis induced liver disease. Galle et al [90] demonstrated that human hepatocytes were extremely sensitive to Fas ligand exposure *in vitro*, with apoptosis being induced in greater than 95% of cells within hours. Further examination of liver biopsy tissue revealed high levels of Fas and Fas ligand mRNA in liver tissue with evidence of ongoing liver damage. Fas ligand was observed to occur in areas of intense lymphocytic infiltrate in patients with hepatitis B suggesting a role for T cell mediated induction of apoptosis and hence liver damage. T-cell mediated Fas/Fas ligand induced apoptosis may also play a role in the deletion of virus infected peripheral lymphocytes in HIV [91].

1.3.3 The Bcl-2 Family of Proteins in the Regulation of Apoptosis

The Bcl-2 family of proteins as mentioned, play a key intermediary role between the cell surface receptors and the inhibition or activation of the caspases. The Bcl-2 gene was first identified in 1985 by Tsujimoto et al [76] in their study of cells from patients with B-cell follicular lymphomas. It was discovered that the translocation characteristic of this type of lymphoma, t(14;18) caused interruption of the gene sequence coding for Bcl-2. Later studies demonstrated that B-cells expressing the Bcl-2 protein were resistant to apoptosis associated with specific cytokine withdrawal [92]. It thus became clear that the expression of this protein was associated with the inhibition of apoptosis and possibly contributed to the malignant nature of the cell line.

Over the subsequent years it became evident that Bcl-2 was only one protein in a large family of approximately a dozen proteins that regulate apoptosis at an intracellular level. Some of the proteins within the family were shown to have a pro-apoptotic affect on cell survival and others were demonstrated to protect against apoptosis. The family of proteins have been divided into three groups, based on structural similarities and functional criteria [77]. Group 1 members posses anti-apoptotic activity, whereas members of group II and III promote cell death. All members of the Bcl-2 family share at least some sequence homology. For example members of group I, Bcl-2 and Bcl-X_L, share at least four short homologous genetic sequences, labelled BH1 through to 4. Members of group II such as Bax and Bak share only the BH1, 2 and 3 sequences with the group I proteins and members of group III such as Bid and Bik, share only the BH3 sequences with group I.

It is the interplay between these proteins within the cell that determines the fate of a cell. It is proposed that the Bcl-2 family of proteins interact to form heterodimers between pro-apoptotic and anti-apoptotic members. The ultimate fate of the cell depends on the overall balance between these two stimuli. The homologous domains described earlier have been demonstrated as the site of interaction between the proteins to form the heterodimers. For example Yin et al [93] were able to show that mutations within the BH1 and BH2 sequences blocked the formation of heterodimers between Bax and Bcl-2. The action of Bcl-2 as an anti-apoptotic protein without heterodimerisation with Bax was shown to be diminished, suggesting that Bcl-2 activity is dependant upon interaction with Bax.

Similarly, studies have demonstrated the requirement of the BH3 region in pro-apoptotic proteins for the induction of cell death. Zha et al [94] demonstrated the pro-apoptotic protein Bad was dependant upon an intact BH3 region to heterodimerise with both Bcl-2 and Bcl-X_L and induce cell death. Bcl-2 can be induced to act as a pro-apoptotic protein if a sequence from the BH3 region of Bax is inserted in place of the corresponding sequence in Bcl-2 [95].

The structure of only some of the proteins in the Bcl-2 family have been characterised. For example, Muchmore et al [96] have demonstrated the three dimensional structure of Bcl-X_L and in doing so have provided some clues as to the proteins mechanism of action. The protein consists of two central hydrophobic helices

surrounded by five amphipathic helices as well as a 60-residue flexible loop. The three homologous regions, BH1, BH2, and BH3 were demonstrated to be in close spatial proximity on the surface of the protein, forming an elongated hydrophobic cleft which is thought to be the binding site of other Bcl-2 family proteins. The 3-D structure of the protein was demonstrated to be similar to that of the pore forming domains of certain bacterial toxins such as diphtheria toxin. This association suggested that the Bcl-2 family of proteins mechanism of action involves the formation of transmembrane channels.

The Bcl-2 family of proteins have been noted to be localised to the outer mitochondrial, outer nuclear and endoplasmic reticular membranes [97]. As mentioned, their mechanism of action at these sites is intimated by their 3-D structure homology with pore forming bacterial toxins. The ability of Bcl-X_L for example to form channels in synthetic lipid membranes was demonstrated *in vitro* by Minn et al [98]. These investigators were able to demonstrate that Bcl-X_L could be inserted into planar lipid bilayers to form ion-conducting channels. The channel was shown to be pH sensitive. Similar observations have been made for other members of the Bcl-2 family, such as Bcl-2 itself and Bax [99, 100].

The precise role these ion-conducting channels play in inducing apoptosis *in vivo* remains undetermined. Hengartner [77], in his recent review of the topic, summarises the three current competing hypotheses. The first suggests that the proteins either in isolation or upon heterodimerisation with other Bcl-2 family members insert into the outer mitochondrial membrane to form channels or large holes. Through these channels proteins may pass into or out of mitochondria to induce apoptosis. The second mechanism suggests that the Bcl-2 family of proteins recruit other outer mitochondrial membrane proteins to create large membrane pores that allow passage of proteins into or out of mitochondria to induce apoptosis. Finally, the third hypothesis suggests that upon creation of mitochondrial membrane pores, the Bcl-2 family of proteins alter mitochondrial physiology such that the organelle swells, resulting in rupture of the outer membrane and release of intermembrane proteins into the cytosol, thus inducing apoptosis.

These three theories all attempt to explain the passage of cytochrome *c* from within the mitochondria to the cytosol, an almost universal feature of apoptotic cell

death. With the passage of cytochrome *c* into the cytosol there is further activation and recruitment of various proteins within the caspase system to induce irreversible cell death.

1.3.4 The Caspase System in the Induction of Apoptosis

As mentioned, the caspase system incorporates a large group of cysteine-containing proteases that are the final step in the "execution" of a cell by apoptosis. As the final step in the induction of apoptotic cell death, blocking caspase activity has been demonstrated to inhibit tissue injury by apoptosis in a variety of experimental models. For example, Mocanu et al [101] were able to demonstrate the affect of inhibiting a variety of caspases in a rat model of cardiac ischaemia-reperfusion injury. By selectively inhibiting several of the caspases, they were able to significantly limit the amount of tissue infarction in their model, specifically by a reduction in cardiac myocardiocyte apoptosis.

To date 13 mammalian caspases have been identified with 11 of them occuring in humans. Caspases are synthesised as inert zymogens, requiring cleavage of a pro-domain for activation. Once activated, the caspases selectively cleave a specific set of target proteins resulting in downstream protein activation or inactivation.

There are two sub-classes of caspases that have been identified based on phylogenetic analysis: the caspase 1 subfamily and the caspase 3 subfamily. The caspase 1 subfamily which includes caspases 1, 4, 5 and 13 are thought to be predominantly involved in the control of inflammation. The caspase 3 subfamily which includes caspases 3 and 6-10 are involved primarily in the induction of apoptosis [102].

As mentioned, caspases are usually present within cells as inactive zymogens. An N-terminal pro-domain is usually attached to the active caspase sequences, p10 and p20. Cleavage of the pro-domain results in activation of the p10 and p20 subunits. Cleavage can occur by a number of mechanisms. Firstly, upstream caspases activated by other mechanisms can induce cleavage of the pro-domain in downstream caspases to induce activation. For example as discussed, activation of the cell surface receptor Fas can lead, via a complex sequence of steps, to activation of caspase 8. As demonstrated by Stennicke et al [103] *in vitro*, caspase 8 induces activation of caspase 3 by cleavage of the pro-domain. Caspase 3 can in turn serve to activate caspase 6 [104], thus

demonstrating what Hengartner [77] termed the "caspase cascade". Both caspase 3 and 6 are central to the induction of the cellular changes associated with apoptosis.

Caspases can also be self-activated by the recruitment of multiple molecules of the same procaspase in a confined intracellular space. This "induced proximity" model for caspase 8 activation was first demonstrated by Muzio et al [105]. They were able to show that caspase 8 possesses intrinsic enzymatic activity that allows self-activation by cleavage of pro-domains. Similar mechanisms of activation have been demonstrated for other members of the caspase family [106].

Finally caspase activation can take place by association of the caspase with a regulatory subunit. Caspase 9 for example is activated by the binding of two protein subunits; cytochrome *c* and Apaf-1. Cytochrome *c* as mentioned is an electron transport protein that is released from mitochondria into the cytosol under the control of the Bcl-2 family of proteins. Apaf-1 is a cytoplasmic protein that binds released cytochrome *c* [107] to then undergo a conformational change that allows binding and activation of procaspase 9 [108]. Without the attached subunits, activated caspase 9 has little intrinsic proteolytic activity.

Once activated, the caspase 3 subfamily of caspases target a variety of proteins to induce cellular apoptosis. These target proteins are usually involved in the maintenance of cell survival and once activated or inactivated cell death occurs. Also by activation of otherwise normally benign pro-apoptotic proteins, caspases can induce active cell membrane and DNA damage to result in cell death.

For example, Wylie [109] demonstrated early in the study of apoptosis that apoptotic cells are characterised by the presence of multiple DNA fragments with lengths of approximately 180 base pairs. He postulated that with the induction of apoptosis, an intracellular endonuclease is activated to cleave genomic DNA and induce the chromatin condensation observed in these cells. Latter experiments by Nagata [110] demonstrated the presence of a specific DNase that cleaves chromosomal DNA in response to caspase 3 activation.

Other target proteins of the caspase family that induce apoptosis include molecules that maintain the cellular cytoskeleton. The integrity of the cellular cytoskeleton is dependant upon a cell's interaction with its neighbouring cells as well as with specific extracellular matrix proteins. Activation of caspase 3 has been

demonstrated to cleave the cytoskeletal protein gelsolin [111] resulting in disruption of actin filaments and induction of nuclear fragmentation *in vitro*, mimicking changes observed in apoptotic cells *in vivo*.

More than 100 target proteins have been identified that are activated or inactivated by pro-apoptotic caspases [102]. To date many of these remain poorly characterised, but the sheer number of identified substrates hints at the complexity of the processes involved in this final step towards cell death.

1.4 Apoptosis in Glomerulonephritis

1.4.1 Introduction

In the last thirty years it has become clear that apoptosis plays a central role in all aspects of cell biology. From early embryogenesis, throughout an organism's life, in disease states and in death the process of programmed cell death has been demonstrated to occur. As highlighted by Heemels [112] in a recent editorial, "optimum human body maintenance means that about 10 billion cells die on a normal day just to counter the numbers of new cells that arise through mitosis".

Whilst the study of apoptosis in organ development and organ maintenance is crucial to understanding normal cell biology, a clear understanding of the role of apoptosis in tissue injury and diseased states has significant implications for therapeutic intervention. As many diseased states arise from either uncontrolled tissue proliferation or from injury-induced tissue atrophy it is clear that those processes that regulate cell number and control cell death are pivotal in the understanding of the aetiology of these diseases. Only with this understanding will the development of specific drugs that target these processes be possible.

For example as mentioned, in some B-cell lymphomas, translocation of the Bcl-2 gene to the control region of the immunoglobulin promoter results in B-cell accumulation due to the inhibition of apoptosis. This demonstrated that cancer does not only arise as a result of unchecked cellular proliferation but also as a result of insufficient cellular apoptosis. With this knowledge it has become possible to develop Bcl-2 antisense molecules that block the action of Bcl-2 in some tumours and allow apoptosis to occur. G-3139 is one such Bcl-2 antisense molecule that has been

demonstrated to have some promising effects in reducing Bcl-2 expression in patients with non-Hodgkin's lymphoma as part of phase I clinical trials [113]. Similarly the G-3139 Bcl-2 antisense molecule has demonstrated a role in suppressing tumour activity when combined with more standard chemotherapeutic agents for the treatment of malignant melanoma [114].

Whilst unchecked cellular proliferation is often the cause of end-organ damage in most forms of cancer, organ dysfunction resulting from tissue atrophy and fibrosis is common in other forms of disease. Myocardial infarction for example, results in significant myocardiocyte necrosis but is also accompanied by apoptosis of myocardiocytes particularly at the border zones of infarcted areas [115]. It has been suggested that this infarct border zone of apoptotic cells contributes to the deleterious effects of left ventricular remodelling [116]. Recent work by Zhao et al [117] have suggested that reduction in myocardiocyte apoptosis using endonuclease inhibitors can reduce the area of myocardial infarction and improve contractility in a dog model of cardiac reperfusion injury. Similar observations have been made by Mocanu et al [101] in a rat model of cardiac injury.

As the end-point for most forms of renal disease are small, atrophic kidneys characterised by glomerulosclerosis (glomerular cell deletion and increased mesangial matrix), interstitial fibrosis and tubular atrophy [118], it is highly likely that cell loss through apoptosis plays a pivotal role in the pathogenesis of end-stage renal disease. Whether apoptosis is the *driving force* in the evolution of the end-stage kidney or rather an epiphenomenon that accompanies irreversible tissue injury remains unclear.

Most forms of acute glomerulonephritis are characterised by mesangial proliferation accompanied by glomerular inflammatory cell infiltration. In some forms of glomerulonephritis such as post-infectious glomerulonephritis, there is a often a clear onset of clinical disease associated with proliferative biopsy changes which both completely resolve with disease remission [119]. As will be discussed, it is clear that cellular apoptosis is mostly responsible for the clearance of the inflammatory cells and resolution of the disease process [120]. Efficient clearance of inflammatory cells and the causative agent along with restoration of normal glomerular architecture gives rise to the excellent renal prognosis associated with this form of glomerulonephritis [121].

However it is clear that other forms of proliferative glomerulonephritis are not associated with disease remission and such an excellent prognosis. For example WHO class IV lupus nephritis, a severe form of proliferative glomerulonephritis, is associated with progressive renal failure in up to 83% of patients without treatment [122].

What distinguishes renal outcomes in the various forms of proliferative glomerulonephritis is certainly multifactorial but it is most likely that the ability of the kidney to clear proliferating and infiltrating cells from the glomerulus without the release of inflammatory cytokines or the induction of glomerular scaring is a major determinant. It is clear that efficient apoptosis of infiltrating glomerular cells and the maintenance of glomerular architecture is likely to be central to this process.

Whilst it is tempting to separate acute glomerular injury and its resolution from chronic glomerular injury and the development of glomerulosclerosis and interstitial atrophy, such separation most likely oversimplifies the complex processes occurring in glomerular injury. Such separation may be helpful in animal models to distinguish the unique features in the pathogenesis of the two processes, but in human disease it is likely that the two occur simultaneously. Furthermore, the interplay between acute injury and chronic injury is likely to hold the key to disease progression, particularly in IgA nephropathy where the renal outcome is so variable.

1.4.2 Apoptosis in the Resolution of Glomerular Inflammation

Many forms of glomerulonephritis are characterised by an increase in glomerular cell number, either proliferation of resident glomerular cells such as mesangial cells or by infiltration of the glomerulus by inflammatory cells. The stimuli that incite this increase in cell number in human disease are varied and poorly understood as has been discussed with regard to IgA nephropathy.

However, perhaps of more importance in the pathogenesis of chronic renal damage in glomerular disease is not the *stimulus* for glomerular cell proliferation but rather the mechanisms for resolution of this proliferation. John Savill, in a recent review of apoptosis in glomerular disease [123], goes so far as to suggests that "glomerular cell proliferation is a beneficial response, that promotes healing and restitution of the injured glomerulus". He sites the Thy1.1 animal model of mesangial cell proliferation as evidence for this theory. This rat model, developed by Bagchus et al [124] in 1986, is

thought to be the animal model that most closely mimics human IgA nephropathy in the mesangial cell response elicited by antibody stimulation.

The model involves infusion of monoclonal anti-rat Thy1.1 antibody into a rat by single intravenous injection. The Thy1.1 antigen is expressed by glomerular mesangial cells and infusion of the antibody induces complement mediated lysis of mesangial cells. Within 4 days of the antibody mediated lysis of mesangial cells, marked glomerular hypercellularity is observed with glomerular crescent formation by day 14. The cellular proliferation is observed to last up to 50 days, with eventual restoration of normal glomerular structure. Immediately after the injection of antibody, massive proteinuria develops which returns to normal after 3 weeks.

Whilst the Thy1.1 animal model mimics human IgA nephropathy in that it induces a proliferative glomerulonephritis, the model still differs from human IgA nephropathy in a number of fundamental ways. Firstly unlike IgA nephropathy, the Thy1.1 model *does not* involve glomerular deposition of IgA as the stimulus for mesangial cell proliferation. As already discussed, mesangial IgA deposition is the only consistent feature observed in human IgA nephropathy. Secondly, the Thy1.1 model involves a single stimulus followed by a transient episode of glomerulonephritis that resolves completely with the passage of time. IgA nephropathy on the other hand, involves relapsing/remitting glomerulonephritis and in some patients, progressive renal failure. Chronicity and renal damage can be induced in the Thy1.1 model by repeated antibody exposure [125] but this tends to be rapidly progressive in *all* exposed rats.

As such it should be noted that the Thy1.1 animal model of glomerulonephritis at best only approximates one aspect of human IgA nephropathy. Despite this, experiments using this model of the disease have revealed much about the role of the mesangial cell in the pathogenesis of proliferative glomerulonephritis.

John Savill and his colleagues in the University of Nottingham, England have studied the Thy1.1 model of glomerulonephritis extensively. They and others have demonstrated the central role mesangial cell apoptosis plays in the resolution of glomerular hypercellularity in the Thy1.1 model of glomerulonephritis. Their first task was to demonstrate that the program of apoptosis was available to isolated mesangial cells. In 1994, Baker et al [126] from this group were able to clearly demonstrate that under specific conditions cultured mesangial cells did indeed exhibit all the

morphological changes associated with cellular apoptosis. Cultured mesangial cells from humans, rats and pigs were deprived of the growth factors present in fetal calf serum and in other culture media such as insulin, selenium and transferrin. In all of the growth factor deprived cultures, there was a progressive increase in the proportion of cells displaying the morphological changes of apoptosis.

To further confirm their observations, cultured mesangial cells were then exposed to the toxin cyclohexamide, a potent inhibitor of protein synthesis. With exposure to this toxin *and* deprivation of growth factors, the proportion of mesangial cells observed to undergo apoptosis was greatly increased. Thus it was clear that mesangial cells *in vitro* were able to be induced to undergo apoptosis when "stressed" by deprivation of growth factors or by exposure to specific toxins.

Baker et al [126] in the same publication went on to demonstrate that mesangial cells could also be observed to undergo apoptosis *in vivo* based upon observations made using the Thy1.1 rat model of glomerulonephritis described earlier. Rats were exposed to the Thy 1.1 antibody to induce mesangial proliferation and then sacrificed at various time points, up to 42 days post exposure to the antibody. Over the course of the 42 days, kidneys were examined for mitotic figures as well as for cells displaying the morphological features of apoptosis.

They observed that by day 10, glomerular cell number had reached its maximum and by day 42 had returned to normal. As anticipated, there was a gradual increase in mitotic figures within glomeruli peaking at day 5. Most importantly it was observed that with the fall in total glomerular cell number after day 10, there was a corresponding increase in apoptotic cell number. This increase in apoptotic cell number was observed to initially correspond with the increase in mitotic cell number. However, as mitotic cell number returned to baseline, apoptotic cell number continued to be significantly elevated well past the forty second day. The authors concluded that apoptosis clearly does take place in glomerulonephritis and was the major mechanism responsible for removing extraneous mesangial cells. The process of apoptosis was thought to be responsible for restoring normal glomerular cell number as well as normal glomerular function.

Similar observation to these were made independently by Shimizu et al [127] in 1995 using the same rat model of Thy 1.1 glomerulonephritis. They demonstrated the

same corresponding peaks of proliferating cell number with apoptotic cell number again suggesting that clearance of infiltrating inflammatory cells as well as superfluous mesangial cells was dependant upon the process of apoptosis.

Baker and Shimizu have clearly demonstrated that apoptosis plays a role in restoring cell number in animal models of proliferative glomerulonephritis. Unfortunately to demonstrate the same for human glomerular disease is difficult given the need for multiple tissue samples to be taken over time. As human glomerulonephritis is most often diagnosed with a single renal biopsy specimen observations must be based on this "snap-shot" of disease. With diseases such as IgA nephropathy, that may take years to develop, this is obviously not ideal but unavoidable as serial renal biopsies in human subjects are not justifiable as enlightening as they may be. This difficulty is highlighted by Savill in a recent review of human post-infectious glomerulonephritis [120]. There he strongly contends that resolution of this disease is dependant upon efficient clearance of infiltrating neutrophils and macrophages from the glomerulus but can only provide evidence for this based upon animal models of disease.

Despite this several authors have published observational works that document the presence or absence of apoptotic cells in several forms of proliferative glomerulonephritis. For example, Wagrowska-Danilewicz and Danilewicz [128] examined human renal biopsies from a variety of conditions for the presence of apoptotic cells as judged by the TUNEL technique that shall be described later. They noted that there were significantly greater numbers of glomerular apoptotic cells in biopsies from patients with IgA nephropathy compared with patients with non-proliferative glomerulonephritis, such as minimal change disease. The number of glomerular apoptotic cells observed in the IgA biopsies correlated strongly with the total number of glomerular cells in the renal tissue. Of note interstitial and tubular apoptotic cells, whilst observed for all patient groups, did not differ statistically between patients with proliferative and non-proliferative glomerulonephritis.

Similarly Soto et al [129] examined human biopsy samples for apoptotic cells as well as for proliferating cells in a variety of glomerular diseases. They too observed significantly increased apoptotic cell numbers in biopsies from patients with proliferative glomerulonephritis. In particular patients with IgA nephropathy and post-streptococcal glomerulonephritis had the highest number of glomerular apoptotic cells.

This was shown to correlate significantly with proliferating cell number. Both these observational studies support the hypothesis proposed by Baker et al [126], that cell number in proliferative glomerulonephritis is controlled by apoptosis. However, the problem of "snap-shot" human biopsy studies alluded to previously, remains.

Furthermore, both Soto et al [129] and Wagrowska-Danilewicz and Danilewicz [130] observed that in WHO Class IV lupus nephritis there was an unexpected paucity of apoptotic cells when compared to other forms of proliferative glomerulonephritis such as IgA nephropathy. There was no correlation between proliferative cell number and apoptotic cell number as observed with IgA nephropathy and the ratio of proliferating cells to apoptotic cells was significantly higher in patients with lupus nephritis. This all suggested that unlike proliferative glomerulonephritidies such as IgA nephropathy, lupus nephritis is characterised by a deficiency in apoptotic clearance of proliferating cells and thus bears a much worse prognosis due to unchecked glomerular hypercellularity and its affect on glomerular function. This theory of deficient apoptosis in lupus as an underlying mechanism of disease is supported by observations made by other authors [131].

1.4.3 Apoptosis in Glomerulosclerosis

From what has been discussed, it is highly likely that apoptosis of proliferating glomerular cells allows for complete resolution of several forms of human glomerulonephritis such as post-infectious glomerulonephritis. However what is clear from studies of the natural history of most other forms of proliferative glomerulonephritis is that glomerular proliferation is often the fore-runner of glomerulosclerosis and renal failure. As has been discussed already with regards to IgA nephropathy, glomerulosclerosis is the strongest predictor of disease progression in almost all published studies. Furthermore heavy proteinuria, the hallmark of glomerular dysfunction is also strongly linked to disease progression.

At the same time as Savill and his group were making their observations on apoptosis in the resolution of glomerular hypercellularity, Sugiyama et al [132] suggested that apoptosis was in part involved in the cellular deletion characteristic of glomerulosclerosis. In a rat remnant kidney model which is characterised by the rapid development of glomerulosclerosis, proteinuria and renal failure, Sugiyama and his

colleagues examined timed renal biopsies for the presence of apoptotic cells. They noted a significant increase in the number of glomerular apoptotic cells after 5/6 nephrectomy compared to controls. Similarly, there was an increase in tubulointerstitial apoptotic cell number in the nephrectomised rats compared with controls. Most significantly the apoptotic cells were observed to appear within areas of glomerulosclerosis. Human biopsy samples were then examined for apoptotic cells. Biopsy samples from patients with IgA nephropathy and lupus nephritis in particular were assessed. Unlike previous authors who demonstrated a significant correlation of proliferating cell numbers with apoptotic cells, Sugiyama et al observed a significant correlation between glomerulosclerosis and apoptotic cell number in these diseases. This paper suggested a totally different role for glomerular cell apoptosis, implying that it was the major mechanism for the development of glomerulosclerosis and tubulointerstitial injury characteristic of progressive renal injury.

Shimizu et al [133], who as mentioned had concurred with observations made on the role of apoptosis in the resolution of glomerular proliferation, also published a paper that described the role of apoptosis in the development of sclerotic lesions in experimental crescentic glomerulonephritis. In their study, crescentic glomerulonephritis was induced in rats using a single dose of anti-glomerular basement membrane (GBM) antibody. This induced a severe acute crescentic glomerulonephritis in the rats with associated proteinuria and progressive renal failure. Animals were sacrificed over the subsequent 8 weeks to examine the serial glomerular changes that ensued, paying particular attention to the degree of apoptosis induced along the time course of disease.

From Day 5 to Week 4, glomerular changes included the formation of crescents accompanied by an influx of leucocytes, mesangial proliferation and accumulation of mesangial matrix. At this stage there was a decrease in glomerular hypercellularity with ongoing accumulation of extracellular matrix and the proliferative lesions progressed to sclerotic lesions. By Week 8 most of the glomeruli had become sclerotic with marked associated interstitial atrophy and fibrosis. With regards to apoptotic glomerular cells, initially these were observed in the necrotizing and proliferating regions within the glomerulus consistent with resolving glomerular inflammation. However after Week 4, with the development of glomerulosclerosis, apoptotic cell numbers increased

significantly. These cells were once again observed to occur within sclerotic regions of the glomerulus. As cellular crescents transformed to fibrous crescents over time, apoptotic cells were observed to occur in association with the fibrotic lesions. The authors concluded that apoptosis was the basic mechanism involved in the resolution of glomerular proliferation *as well as* in the development of glomerulosclerosis.

In support of this theory, Yang et al [134] were recently able to demonstrate the anti-glomerulosclerotic affect of caspase 3 blockade in the development of glomerulosclerosis in experimental crescentic glomerulonephritis. A rat model of anti-GBM antibody induced glomerulonephritis was again used as a model for human disease. The pan caspase inhibitor Boc-Asp (OMe)-fluoro-methyl-ketone (B-D-FMK) was infused directly into the kidney of rats after exposure to the nephrotoxic antibody. One group of rats was exposed to the caspase inhibitor for the first 7 days post disease induction and a second group was treated from Day 15 until Day 43.

Reduced glomerulosclerosis was observed in both early and late treated groups compared to untreated controls. This was accompanied by a significant reduction in apoptotic cell number in rats treated early with the caspase inhibitor. Rats treated late with the inhibitor had similar numbers of glomerular apoptotic cells compared to untreated controls but a reduction in tubulointerstitial apoptosis and fibrosis. Overall both treated groups had significantly less proteinuria than untreated controls. Disappointingly renal function was not better preserved in rats treated early with the caspase inhibitor.

The role of apoptosis in the progression of glomerulosclerosis is highlighted by studies of diabetic nephropathy, a condition that is not associated with glomerular proliferation. This form of glomerulopathy is characterized by a nodular increase in extracellular matrix and glomerulosclerosis with associated increase in the width of glomerular basement membrane. There is no associated glomerular infiltration or mesangial proliferation and thus apoptosis observed in association with this condition must be directly related to the development of the sclerotic changes.

Animal models of diabetes such as the streptozotocin-induced diabetic model have been utilized by some authors to investigate the role of apoptosis in the development of the characteristic diabetic glomerulopathy. For example Pesce et al [135] induced diabetes in rats using streptozotocin and examined renal tissue for

apoptotic cells at 2 months and at 6 months. Early in the development of diabetic nephropathy there were few apoptotic cells observed within glomeruli. However after 6 months of diabetes, significantly higher numbers of apoptotic cells were observed within glomeruli with an associated decrease in total glomerular cell number. This suggested that cell deletion and the development of diabetic glomerulosclerosis was mediated by apoptotic cell death. Alternatively, those factors that induced glomerulosclerosis in diabetic nephropathy also induced glomerular cell apoptosis. Pro-fibrotic cytokines such as TGF-β, released in diabetic nephropathy, may stimulate both cellular deletion and the increased matrix deposition.

Apoptosis associated with glomerulosclerosis and interstitial atrophy has been demonstrated to occur in other forms of non-proliferative glomerulopathy. For example Ying, Wang and Saunders [136] were able to demonstrate increased apoptosis in the development of experimental hypertensive glomerulosclerosis. Enhanced apoptosis also appears to play a role in the development of renal cysts in a canine model of polycystic renal disease [137].

Thomas et al [138] expanded upon observations made by Sugiyama et al [132] in the rat 5/6 nephrectomy model of chronic renal failure, focusing on the role of apoptosis in the tubulointerstitial compartment rather than the glomerulus. They also observed a similar increase in glomerular apoptosis as the disease progressed with associated glomerulosclerosis. However the highest number of apoptotic cells was observed in the tubulointerstitial compartment of diseased kidneys. By Day 120 of disease, the number of glomerular apoptotic cells was 10 times that of controls with the number of tubular apoptotic cells 26 times that of controls. There was a strong correlation between tubulointerstitial fibrosis scores and apoptotic cell number. Apoptotic cell number also correlated significantly with the degree of renal failure and proteinuria. Others authors have confirmed this increase in tubulointerstitial apoptosis in other models of chronic renal failure associated with tubulointerstitial fibrosis including obstructive uropathy [139] and polycystic renal disease [140].

It is clear that apoptosis is a focal point for a variety of different glomerular processes. Resolution of glomerular hypercellularity in proliferative forms of glomerulonephritis is mediated through programmed cell death to restore glomerular

structure and function. In glomerulosclerosis and tubulointerstitial atrophy, the cell deletion that is the hallmark of these conditions is also mediated by apoptosis. Whilst complete resolution of glomerulonephritis is rare in human disease, glomerulosclerosis and tubulointerstitial injury is a relatively common end-point for most progressive forms of glomerulonephritis. Inhibition of apoptosis under these circumstances may inhibit renal injury and preserve renal function. In order to achieve this, a clear knowledge of the factors that stimulate and inhibit cellular apoptosis in human disease is necessary.

1.4.4 Regulation of Glomerular Apoptosis

Apoptosis per se is unlikely to be the driving force behind the glomerular and tubulointerstitial changes observed in renal disease states. Rather it is likely that a variety of stimuli and cytokines are expressed in excess or are deficient such that apoptosis occurs as a secondary phenomenon. As the study of apoptosis expands, so too does the list of pro-apoptotic stimuli. As summarized by Savill [123] the list of stimuli reported to induce mesangial cell apoptosis alone stands at twenty. (Table 1.3) The list is divided into non-specific and specific stimuli that *in vitro* have been demonstrated to induce mesangial cell apoptosis but as Savill himself reports, the relevance of these stimuli to disease process remains unclear.

Non-Specific Stimuli	Specific Stimuli
Serum starvation	Anti Thy1.1 antibodies
Detachment	Anti Fas antibodies
Shear stress	IL-1α
Hydrostatic pressure	IL-1β
DNA damage	C1q
Reactive oxygen species	Anti-dsDNA antibodies
Ionizing radiation	LDL
Cytotoxic drugs	Lovastatin
	Nitric oxide
	Superoxide
	Cyclic AMP

Table 1.3 Stimuli reported to trigger mesangial cell apoptosis in culture, from [123].

Several studies have attempted to demonstrate a role for the various cytokines implicated in glomerular and tubulointerstitial disease. Many of these studies explore the expression or up-regulation of a single cytokine or receptor in human and experimental models of glomerulonephritis, a practice which may oversimplify the complex interactions that are likely to occur in human disease between a myriad of apoptotic regulators.

1.4.4.1 Fas and Fas-Ligand in the Induction of Apoptosis in Renal Disease

Gonzalez-Cuadrado et al [141] were able to demonstrate the affect of anti-Fas antibodies on cultured mesangial cells. Fas was shown to be present on the surface of human mesangial cells, with increased expression on exposure to interferon gamma (IFNγ). Anti-human Fas antibody was clearly demonstrated to induce apoptosis of mesangial cells, in a dose dependant response. As expected pre-treatment with IFNγ

increased the cytotoxic affect of the antibody while exposure of the mesangial cells to IFNγ alone had no affect.

Fas-ligand is a tumour necrosis factor-like cytokine that is expressed by T lymphocytes and mediates T cell toxicity [142]. It has also been demonstrated to occur in high levels in human monocyte/macrophages [143]. Both these leukocyte types are observed in human glomerular disease, particularly proliferative glomerulonephritis. The union of the ligand with its receptor has been demonstrated in other tissues to be a potent inducer of cellular apoptosis [90]. Thus it is highly likely that infiltrating leucocytes may be one mechanism of Fas-ligand–Fas union and the induction of glomerular apoptosis in human disease.

Gonzalez-Cuadrado et al [144] were able to demonstrate this effect on mice kidneys (as well as livers) by a single intra-peritoneal injection of Fas-ligand. They found that the Fas-ligand injection proved fatal in 30% of mice at 24 hours. Mesangial cell injury was observed at 3 hours with clear depletion of mesangial cells and increase in mesangial cell matrix. Several techniques were utilized to demonstrate the presence of a fivefold increase in mesangial cell apoptosis as soon as 3 hours after injection of Fas-ligand. This rate of apoptosis continued to increase with time, peaking at 24 hours. Exposed mice were noted to develop haematuria and proteinuria with the onset of glomerular injury, as well as liver failure associated with hepatocyte apoptosis.

As cell deletion is a major feature of tubulointerstitial disease associated with chronic renal injury, other authors have focused on the role of Fas/Fas-ligand on the induction of renal tubular atrophy in animal models of chronic renal failure. Schelling and Cleveland [145] for example, using two mice models of chronic renal injury, examined renal sections for tubular apoptosis. Tubular cell apoptosis was significantly increased in chronic renal injury models compared to age matched controls. This increase in tubular apoptotic cell number persisted for up to 26 weeks in the diseased mice. They then went on to demonstrate *in vitro* renal tubular cell expression of Fas-ligand as well as Fas, suggesting that interactions between the two in adjacent tubular cells may give rise to the observed tubular apoptosis.

In support of these observations in animal models of renal disease, several authors have attempted to document the presence of Fas and Fas-ligand in human renal disease. Takemura et al [146] examined renal tissue for the expression of Fas from

eighty patients with a variety of glomerulonephritidies using normal renal biopsies as controls. They demonstrated significant increase in glomerular Fas expression in lupus nephritis and Henoch-Schönlein purpura compared to normal controls. With dual labelling techniques they were able to show that Fas expressing cells were for the most part mesangial cells and to a lesser extent infiltrating leucocytes. There was a strong correlation between Fas expression and glomerular apoptosis.

Sano et al [147], rather than examine renal tissue for Fas and Fas-ligand expression, measured *serum* levels of soluble Fas and Fas-ligand from patients with a variety of glomerulonephritidies. Although serum levels of soluble Fas-ligand were not found to be elevated in any of the patients, serum levels of Fas were significantly elevated in patients with IgA nephropathy and mesangiocapillary glomerulonephritis. This they found did not correlate positively with the number of apoptotic cells within glomeruli, suggesting an alternative role for soluble Fas in disease progression.

Finally of interest, Bhaskaran et al [148] were recently able to demonstrate the role angiotensin II may play in the development of tubulointerstitial injury. They were able to demonstrate that angiotensin II promoted tubular apoptosis through enhanced expression of tubular Fas and Fas-ligand in cell culture. This enhanced degree of apoptosis was blocked by the presence of angiotensin receptor antagonists. This suggests that some of the demonstrated clinical benefits of these agents, particularly in renal disease, may relate to an anti-apoptotic affect.

1.4.4.2 Bcl-2 Family of Proteins in the Regulation of Apoptosis in Renal Disease

As mentioned the Bcl-2 family of proteins act as "gate keepers" to the induction of apoptosis at an intracellular level via their role on the activation of the caspase group of enzymes. Whether a pro-apoptotic or anti-apoptotic signal is transferred to the caspases depends upon the balance between the pro and anti apoptotic members of the Bcl-2 family and the interactions that take place between them.

Ortiz et al [149] were able to demonstrate the role of the Bcl-2 family of proteins in a mice model of acute renal failure. They measured expression of the pro-apoptotic genes Bax and Bcl-X_S as well as the anti-apoptotic molecules Bcl-2 and Bcl X_L. Within 24 hours of inducing acute renal failure with high doses folic acid, there developed significant tubular apoptosis not present in control mice. Of note early in the disease

process they were able to show in increase in pro-apoptotic mRNA expression in the form of Bax and Bcl-X_S with a corresponding decrease in the expression of the anti-apoptotic Bcl-2. Expression of Bax was noted to be restricted to vascular smooth muscle cells and Bcl-2 to tubular epithelium. No significant staining was noted in the glomeruli with any of the antibodies. The authors conclude that the balance demonstrated in this model in favour of apoptosis is responsible for the cellular changes observed.

Similarly Ying et al [136] in their rat model of hypertensive nephrosclerosis measured mRNA expression of the same group of genes in diseased states and normal controls. With the increased rate of glomerular and tubular apoptosis observed as the disease progressed, there was commensurate rise in expression of the pro-apoptotic genes Bax, Fas and Bcl-X_S. In order to identify whether tubular or glomerular cells were responsible for this up-regulation in pro-apoptotic gene expression, cell lysates from sieved glomeruli and tubules were examined independently. Both tissue compartments were observed to express increased levels of these genes compared to normal controls, suggesting a role for these genes in both glomerular as well as tubular apoptosis.

Several authors have attempted to characterize the distribution of the Bcl-2 family of proteins in human glomerular disease. Yoshimura et al [150] examined biopsies from 19 patients with a variety of glomerular disease including 12 cases of IgA nephropathy for the expression of glomerular Bcl-2. This was then correlated with a marker of cellular proliferative injury. They observed an increase in mesangial cell Bcl-2 expression in patients with IgA nephropathy compared to controls with a correlation between Bcl-2 and glomerular proliferation. The authors suggested a role for Bcl-2 in the amplification of glomerular proliferation in IgA nephropathy.

Other authors do not concur with Yoshimura et al on the glomerular expression of Bcl-2 in proliferative glomerulonephritis. Nakopoulou et al [151] in their study of the expression of Bcl-2 in various forms of glomerulonephritis did not detect any Bcl-2 expression within glomeruli, both normal and diseased. They did however observe significantly higher Bcl-2 expression in the proximal convoluted tubules in cases of proliferative glomerulonephritis compared to non-proliferative renal disease. Of-note, IgA nephropathy accounted for 30% of their patients with proliferative disease.

Finally, Yoshimura et al [152] did further analysis of human renal biopsy samples, on this occasion utilizing *in situ* hybridization techniques to examine for Bax

expression. A repeat analysis of glomerular expression of Bcl-2 was also undertaken using immunohistochemistry. Once again they demonstrated glomerular mesangial expression of Bcl-2 correlating with proliferative markers in patients with proliferative glomerulonephritis compared to non-proliferative disease. Bax expression was observed to be localized to the mesangium and correlated with matrix expansion and Type IV collagen deposition, suggesting a role for Bax in glomerular apoptosis and the development of glomerulosclerosis.

These studies in human renal disease are revealing in that they suggest a role for the Bcl-2 family of proteins in the pathogenesis of renal disease. However examining tissue for only one or two of the family of proteins is likely to lead to oversimplification of this role.

1.4.4.3 Proteinuria in the Induction of Apoptosis in Renal Disease

As has already been discussed, proteinuria is one of the strongest independent predictors of disease progression for IgA nephropathy as well as for most forms of glomerular disease [153]. This association is independent of the degree of observed glomerular injury and thus it is likely that proteinuria *per se* acts as a nephrotoxin.

The nature of the nephrotoxicity of proteinuria remains poorly understood. Kees-Folts et al [154] demonstrated that urine from proteinuric rats contained a macrophage chemotactic factor. They suggested that tubular metabolism of albumin-borne fatty acids gives rise to a novel lipid that accumulates within the interstitium and acts as a chemoattractant for macrophages.

Others have suggested that albumin *itself* is directly toxic to the proximal convoluted tubule, inducing pro-inflammatory cytokine release from tubular cells and initiating the process of interstitial infiltration, fibrosis and scarring [155]. Other potential cytotoxic proteins that occur in high concentrations in the proximal convoluted tubule in proteinuric states include transferrin, iron, low-density lipoprotein and complement proteins [156].

Regardless of the culprit protein or lipid in proteinuric tubulotoxicity, some authors have demonstrated increased glomerular and proximal convoluted tubule apoptosis in association with proteinuria. For example Thomas et al [157], in their rat model of protein-overload proteinuria, examined renal sections for apoptotic cells. These were noted to be significantly increased in glomeruli from proteinuric animals

compared to controls. Similar observations were made by Thomas et al [158] with regard to tubular and interstitial cell apoptosis in this proteinuric model. Associated with tubular apoptosis, Thomas et al observed a significant increase in proximal tubular cell proliferation, less marked than the degree of apoptosis. The authors conclude that proteinuria through unknown mechanisms induce tubular cell apoptosis *and* proliferation with the overall balance favouring apoptosis and tubular atrophy.

In sharp contrast to what has previously been suggested, other authors have suggested that albumin acts to inhibit tubular apoptosis at least *in vitro*. Iglesias et al [159] for example, were able to show that bovine serum albumin protected cultured tubular cell from apoptosis in growth factor deficient medium. The authors went on to suggest that bovine serum albumin is responsible for scavenging reactive oxygen species that accumulate in deficient medium and thus protect against apoptosis.

This apparent discrepancy in the role of albumin as both an inhibitor of apoptosis and an inducer of apoptosis is addressed in a recent editorial by Iglesias and Levine [160]. They suggest that under physiological circumstances albumin contributes to tubular cell homeostasis through its anti-oxidant activity and via the delivery of non-cytokine growth factors. However, in disease states, albumin may act as carrier molecule for other nephrotoxins such as oxidized lipids as well as reactive carbonyl groups.

Many other mechanisms for the regulation of apoptosis in glomerular disease have been suggested. Evidence exists for cytokines such as TGF-β [161] and connective tissue growth factor [162] in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis via induction of apoptosis. Other non cellar mechanisms have also been implicated in the induction of mesangial cell apoptosis and subsequent development of glomerulosclerosis, in particular the disruption of extracellular matrix and mesangial cell interactions [163].

1.4.5 Apoptosis in the Progression of IgA Nephropathy

For such a common disease as IgA nephropathy surprisingly little has been published that specifically examines the role of apoptosis in the progression of the disease. Whilst it is clear that much has been written on the role of apoptosis in animal models of proliferative and sclerosing glomerulonephritis, these models only

approximate human IgA nephropathy and thus extrapolating from animal to human studies may not be appropriate.

Given that the pathogenesis of IgA nephropathy remains unclear as previously discussed, no animal model exists that can truly replicate the disease. Unfortunately, without an accurate animal model of IgA nephropathy, our observations on the disease must come from human studies that rely on small sections of renal tissue taken at a single time point over the course of a disease that may last for many years.

There are certainly some studies that have already been discussed that examine IgA nephropathy amongst other disease entities for the expression of apoptotic cells or some of the regulators of apoptosis. None of these attempts to distinguish between patients with progressive IgA nephropathy and those with benign disease. This factor will certainly influence the significance of observed apoptotic cells and changes in protein expression. These studies also fail to correlate clinical and histological parameters with the degree of apoptotic injury observed on biopsy.

Nevertheless, two studies do attempt to address the role of apoptosis in the progression of human IgA nephropathy. The first by Tashiro et al [164] examines six patients with IgA nephropathy who underwent repeat renal biopsies to examine the correlation between apoptotic cells observed on the first biopsy and clinical and histopathological changes observed at the second biopsy. Three patients with severe renal damage and heavy proteinuria were observed to have apoptotic cells within glomeruli on the first biopsy. No apoptotic cells were observed in patients with mild disease. On repeat biopsy, two out of the three patients demonstrated to have apoptotic cells on first biopsy no longer displayed any apoptotic cells. These two patients demonstrated some degree of improvement in the severity of the renal lesion seen at biopsy. Of note, 4 out of the 6 patients received immunosuppressive as well as antiplatelet and anticoagulant therapy for treatment of the disease, a practice not widely accepted. Despite this, the absence of apoptotic cells in follow-up biopsies in 2 out of the 3 patients suggests possible suppression of apoptosis with treatment. No long term follow-up data is presented for the patients.

The same group had earlier published a much larger series of patients with IgA nephropathy that demonstrated apoptotic cells in 6 out of 11 patients with severe glomerular lesions and only 1 out of 12 patients with mild glomerular damage [165].

Mean levels of proteinuria at the time of biopsy were significantly higher in patients with apoptotic cells on biopsy compared to those without. There was no significant difference in renal function noted between the two groups. All patients were treated with corticosteroids, anticoagulants, antiplatelet agents and ACE inhibitors. Of note at follow-up, those patients with apoptotic cells present at biopsy had a greater reduction in proteinuria with treatment compared to those without. The authors suggested that apoptotic cells within the glomerulus may be an indicator of possible better response to treatment, although no explanation as to why this may be is offered.

In view of the paucity of published literature that specifically examines the role apoptosis may play in the progression of IgA nephropathy and to expand on observations made by Tashiro et al, the candidate undertook the following studies. The first study was to assess renal biopsy samples from patients with IgA nephropathy for apoptotic cells and to correlate this with clinical and histological parameters as well as with disease progression. Subsequent studies were aimed at quantifying the expression (mRNA and product) of some of the protein regulators of apoptosis discussed in this review. In particular Fas, Fas-ligand and some of the members of the Bcl-2 family of proteins were to be measured and their relative expression correlated with progression of disease.

CHAPTER TWO

METHODOLOGY

2.1 Renal Biopsy Samples

Approval for the entire study was gained from The Alfred Hospital Ethics Committee prior to its commencement. In particular the committee authorized the use of spare human renal biopsy tissue for the purposes of this study.

Percutaneous renal biopsies were performed on patients where clinically indicated on the recommendation of their treating renal physician. Prior to the biopsy, written informed consent was received from the patient for the procedure itself as well as for the usage of any excess renal tissue for the purpose of clinical research.

A segment of each renal biopsy was fixed in formalin and embedded in paraffin by the pathology department of The Alfred Hospital for diagnostic purposes. Any remaining tissue, not required for diagnostic purposes, was immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction was performed. Leftover paraffin-embedded renal tissue was stored in the pathology department at room temperature until required.

Once patients were identified as suitable for inclusion in this study, 2µm sections were cut from the paraffin-embedded, stored biopsy tissue and placed on poly-l-lysine coated slides. Slides were baked at 30°C for one hour and then stored at room temperature until required.

2.2 Slide Preparation for Immunohistochemistry and TUNEL

2.2.1 Poly-L-Lysine (PLL) Microscope Slide Adhesive

All microscope slides were cleaned by soaking them in racks in hot, running tap-water with detergent for fifteen minutes. Slides were then air-dried. A solution of poly-l-lysine was made up at a concentration of 1mg/ml.

Poly-L-Lysine	10mg
Reverse osmosis (RO) water	10ml

A small drop (approximately 5µl) of poly-l-lysine solution was placed on each slide and smeared across the slide using another slide. Slides were then air-dried and stored at 4°C to be used within two weeks.

2.3 TUNEL Technique for Paraffin Embedded Human Tissue

2.3.1 Buffers and Solutions for TUNEL technique

2.3.1.1 Ethanol 90%

Ethanol absolute (100%)	900ml
RO water	100ml

2.3.1.2 Ethanol 70%

Ethanol absolute (100%)	700ml
RO water	300ml

2.3.1.3 0.3% Hydrogen Peroxide/Methanol Block

Methanol	400ml
Hydrogen peroxide 30% solution	4ml

Fresh hydrogen peroxide was added to the solution daily prior to use.

2.3.1.4 Milli-Q Water

Milli-Q water was produced by ultrafiltration of RO water through a 0.22µm filter (Milli-Q PF Plus™ water purification system, Millipore Corporation, USA).

2.3.1.5 Phosphate Buffered Solution (PBS) x10 concentrate

Sodium chloride	160g
Disodium orthophosphate	23g
Potassium chloride	4g
Potassium orthophosphate	4g
RO water	2000ml

To make the x1 PBS washing fluid, 200ml of the x10 PBS concentrate was added to 1800ml of RO water.

2.3.1.6 Protease VIII Solution

Protease VIII	15mg
x1 PBS	100ml

2.3.1.7 Reverse Osmosis (RO) Water

RO water refers to tap water purified via reverse osmosis (Milli –RO Plus™ Millipore Corporation, USA).

2.3.1.8 Sodium Azide 10%

Sodium azide	10g
RO water	100ml

2.3.2 Diluents for TUNEL Technique

2.3.2.1 20% Normal Swine Serum

Normal swine serum	2ml
x1 PBS	8ml
Sodium azide 10%	10µl

2.3.2.2 1% Normal Swine Serum

Normal swine serum	100µl
x1 PBS	9.9ml
Sodium azide 10%	10µl

2.3.2.3 5% Normal Human Serum/1% Normal Swine Serum

Normal human serum	500µl
Normal swine serum	100µl
x1 PBS	9.4ml
Sodium azide 10%	10µl

2.3.3 Antibodies and Reagents for TUNEL Technique

Diaminobenzidine metal (DAB) substrate	Pierce, Rockford, IL. USA
DAB stable buffer	Pierce, Rockford, IL. USA
DIG dUTP	Boehringer Mannhein. Germany
DNase enzyme	Boehringer Mannhein. Germany
Rabbit anti-sheep immunoglobulin-peroxidase conjugated (px)	Dako, Gostrup. Denmark
Sheep anti-DIG Fab' Fragments	Dako, Gostrup. Denmark
Swine anti-rabbit immunoglobulin- px	Dako, Gostrup. Denmark
TDT Buffer	Promega, Madison WI. USA
TDT Enzyme	Promega, Madison WI. USA

2.3.4 TUNEL Technique

(N.B All steps were performed at room temperature unless otherwise stated).

Sections were dewaxed in Histosol twice for five minutes and then rehydrated via graded alcohols to running tap water (100% ethanol twice, 70% ethanol, running tap water, five minutes each). This was followed by a five-minute wash in x1 PBS solution.

Slides were then pre-treated in Protease VIII solution for thirty seconds followed by a five-minute wash in x1 PBS solution. With a Pap Pen (Zymed, Southern San-Francisco, CA. USA) sections were then isolated on the slide surface by encircling them with the hydrophobic gel. Positive control sections were then incubated with DNase diluted 1/10 in x1 PBS for twenty minutes while other sections were incubated in x1 PBS only. This was followed by a five-minute wash in x1 PBS. All sections were then

pre-incubated with TDT buffer diluted 1/5 in Milli-Q water for ten minutes and then drained.

In a moist chamber, slides were incubated in TDT enzyme/DIG dUTP at 37°C for sixty minutes as follows –

Section	DIG dUTP (µl)	TDT enzyme(µl)	Dilute TDT Buffer(µl)
Positive control and Test	1	2.5	346.5
Negative control	1	-	349

Table 2.1 Primary layer for TUNEL technique.

Following a five-minute wash in x1 PBS, slides were then incubated in 20% normal swine serum for ten minutes and then drained. Sections were then incubated in a moist box for sixty minutes with sheep anti DIG F'ab fragments diluted 1:200 with 1% normal swine serum. Following a further five-minute wash in x1 PBS, sections were taken through graded alcohols (70% ethanol, 90% ethanol, 100% ethanol x 2 - 10 dips in each) and placed in 0.3% hydrogen peroxide/methanol block for thirty minutes. Slides were then reversed through the graded alcohols (100% ethanol x2, 90% ethanol, 70% ethanol – 10 dips in each) and washed for five minutes in x1 PBS.

In a moist chamber, slides were then incubated for thirty minutes in rabbit anti-sheep immunoglobulin-px diluted 1:100 in 5% normal human serum/1% normal swine serum followed by a five-minute wash in x1 PBS. Slides were next incubated with swine anti-rabbit immunoglobulin-px diluted 1:50 in 5% normal human serum/1% normal swine serum followed by a five-minute wash in x1 PBS.

Slides were then incubated with DAB substrate diluted 1/10 in DAB stable buffer for 30 minutes followed by a ten-minute wash in running tap water.

Slides were next counterstained with Harris' haematoxylin (see section 2.4) and then dehydrated through graded alcohols (70% ethanol, 90% ethanol, 100% ethanol x2 – 10 dips in each). After clearing the slides with 10 dips in histosol twice, a coverslip was applied using Biomount mounting media.

2.3.5 Counting Apoptotic Cells by Light Microscopy

Prior to counting the candidate was blinded to the origin of the biopsy sample to be examined. All interstitial TUNEL-positive cells were counted using a 100 square graticule at x400 magnification. The area of the whole biopsy sample was calculated and apoptotic cells were expressed as the number of TUNEL-positive cells identified/mm^2. Glomerular TUNEL-positive cells were counted at x400 magnification. The total number of glomeruli in the biopsy sample was counted and apoptotic cells were expressed as the number of TUNEL-positive cells/glomerulus.

2.4 Counterstaining for TUNEL and Immunohistochemistry

2.4.1 Solutions for Counterstaining

2.4.1.1 1% Acid Alcohol

HCL	10ml
Ethanol absolute (100%)	700ml
RO water	290ml

2.4.1.2 Harris' Haematoxylin

Haematoxylin	3g
Ethanol absolute (100%)	30ml
RO water	600ml
Aluminium ammonium sulphate	60g
Mercuric oxide	1.5g
Glacial acetic acid	24ml

The haematoxylin was first dissolved in 30ml ethanol (solution A). The RO water was heated and then the aluminium ammonium sulphate was added and stirred until dissolved (solution B). Solution A was then added to solution B with stirring and brought rapidly to the boil. Once boiling, the mercuric oxide was added (solution C). The solution was then cooled rapidly in a bath of running tap water. Solution C was then filtered and the glacial acetic acid was added to make the final solution of Harris' haematoxylin.

2.4.1.3 2% Sodium Bicarbonate

Sodium bicarbonate	20g
RO water	1000ml

2.4.2 Counterstaining Technique

Prior to use, any surface residue was removed from the Harris' haematoxylin by skimming it with filter paper. Slides were then dipped into the Harris' haematoxylin for approximately thirty seconds followed by a wash in running tap water until clear. Slides were then dipped once into 1% acid alcohol followed by 10 dips in running tap water. Slides were then placed into 2% sodium bicarbonate for approximately thirty seconds until sections turned blue. Slides were then rinsed in running tap water and checked under the microscope for the intensity of nuclear staining. If this proved to be insufficient, the process was repeated increasing the time in Harris' haematoxylin.

2.5 Immunohistochemistry for Paraffin Embedded Human Tissues

2.5.1 Buffers and Solutions for Immunohistochemistry

2.5.1.1 10% Gelatin

Gelatin	20g
RO water	200ml

Gelatin was dissolved with heat and the solution was stored at 4°C until required.

2.5.1.2 PBS/0.2% Gelatin Washing Fluid (w/f)

10% gelatin	40ml
RO water	1760ml
x10 PBS	200ml

Gelatin was warmed in a water bath at 37°C until liquefied and then added to the RO water.

2.5.2 Diluents for Immunohistochemistry

2.5.2.1 10% Normal Rabbit Serum

Normal rabbit serum	1ml
x1 PBS	9ml
Sodium azide 10%	10µl

2.5.2.2 1% Normal Rabbit Serum

Normal rabbit serum	100µl
x1 PBS	9ml
Sodium azide 10%	10µl

2.5.2.3 5% Normal Human Serum/1% Normal Rabbit Serum

Normal human serum	500µl
Normal rabbit serum	100µl
x1 PBS	8.4ml
Sodium azide 10%	10µl

2.5.3 Heat-Induced Antigen Retrieval for Immunohistochemistry

2.5.3.1 0.1 M Citric Acid

Citric acid	21.01g
RO water	1000ml

2.5.3.2 0.1 M Sodium Citrate

Sodium citrate	29.41g
RO water	1000ml

2.5.3.3 Working Solution

0.1 M Citric acid	9ml
0.1 M Sodium citrate	41ml
RO water	500ml

The working solution was mixed well with stirring. Dewaxed and rehydrated slides were then placed in a slide-rack inside a heat-resistant container containing 500ml

of the working solution. The solution was brought to the boil on a hot plate and slides were left in the boiling solution for ten minutes. The slides were removed from the hot plate and left in the working solution to cool at room temperature for approximately twenty minutes prior to proceeding with the staining technique.

2.5.4 Antibodies and Reagents for Immunohistochemistry

2.5.4.1 Reagents

Diaminobenzidine metal (DAB) substrate	Pierce, Rockford, IL. USA
DAB substrate stable buffer	Pierce, Rockford, IL. USA

2.5.4.2 Monoclonal Antibodies

All antibodies were tested over a range of dilutions to determine the concentration of antibody that delivered optimal staining. Final concentrations are listed as µg/µl where this information was available or as dilutions of the original supernatants provided by the manufacturer. All antibodies were further tested following a variety of pre-treatments for antigen retrieval. These included digestion with trypsin, digestion with protease and boiling in citrate buffer (see section 2.5.3). The antigen retrieval method that delivered optimal staining is listed under "Pre-treatment" in Table 2.2. For some antibodies the results were optimal without pre-treatment. (Table 2.2)

2.5.4.3 Secondary antibodies

Goat anti-mouse immunoglobulin	Sigma-Aldrich, St. Louis, MO. USA
Goat peroxidase anti-peroxidase	Dako, Gostrup. Denmark
Rabbit anti-goat immunoglobulin	Dako, Gostrup. Denmark

Antibody	Manufacturer	Specificity	Isotype and Optimised dilution	Pre-Treatment
Bax	Zymed Laboratories	Anti-human Bax antigen	IgG1 1:400	Boiling in citrate buffer
Bcl-2	Dako	Anti-human Bcl-2 antigen	IgG1 1:100	Boiling in citrate buffer
Bcl-X_L	Zymed Laboratories	Anti-human Bcl-X_L antigen	IgG2a 1:100	Boiling in citrate buffer
CD3	Novocastra Laboratories	Anti-human T-cell	IgG2a 1:800	Boiling in citrate buffer
CD-68	Dako	Anti-human macrophage	IgG1 1:10,000	Boiling in citrate buffer
Fas L	Transduction Laboratories	Anti-human Fas ligand	IgG1 5µg/ml	Boiling in citrate buffer
IgG1–ve control	Dako	Isotype negative control	IgG1 5µg/ml	N/A
IgG2a-ve control	Dako	Isotype negative control	IgG2 0.018µg/ml	N/A
L26	Dako	Anti-human B-cells	IgG2a 1:5000	Nil
LCA	Zymed Laboratories	Anti –human leucocyte common antigen	IgG1 1:100	Boiling in citrate buffer
TGF-β	Serotec	Anti-human transforming growth factor beta 1 antigen	IgG1 0.25µg/ml	Boiling in citrate buffer
αSMA	Sigma Biosciences	Anti-human α smooth muscle antigen	IgG2a 1:16,000	Nil

Table 2.2 Monoclonal antibodies used in immunohistochemistry.

2.5.5 Immunohistochemistry Technique

(N.B All steps were performed at room temperature unless otherwise stated).

Sections were dewaxed in histosol twice for five minutes and then rehydrated via graded alcohols to running tap water (100% ethanol twice, 70% ethanol, running tap water, five minutes each). This was followed by a five-minute wash in PBS/0.2% gelatin washing fluid (w/f).

At this stage sections that required pre-treatment for antigen retrieval were identified. These sections were boiled in citrate buffer (see section 2.5.3) followed by a five-minute wash in w/f. With a Pap Pen (Zymed, Southern San-Francisco, CA. USA) sections were then isolated on the slide surface by encircling them with the hydrophobic gel.

Sections were then pre-incubated with 10% normal rabbit serum for ten minutes. Slides were then drained and the primary antibody was applied at the described concentrations diluted in 1% normal rabbit serum. Slides were stored in a moist box at 4°C overnight.

The next day, after a five-minute wash in the w/f, the second layer was applied to sections. Slides were incubated with goat anti-mouse IgG diluted 1:200 in 5% normal human serum/1% normal rabbit serum for thirty minutes. Following a further five-minute wash in w/f, sections were taken through graded alcohols (70% ethanol, 90% ethanol, 100% ethanol x2 - 10 dips in each) and placed in 0.3% hydrogen peroxide/methanol block for thirty minutes. Slides were then reversed through the graded alcohols (100% ethanol x2, 90% ethanol, 70% ethanol - 10 dips in each) and washed for five minutes in w/f.

Next the third antibody layer was applied. Slides were incubated with rabbit anti-goat IgG diluted 1:50 in 5% normal human serum/1% normal rabbit serum for thirty minutes. Following a five-minute wash in w/f, the fourth antibody layer was applied. Slides were incubated with goat peroxidase anti-peroxidase diluted 1:60 in w/f for thirty minutes followed by a five-minute wash in w/f.

Slides were then incubated with DAB substrate diluted 1/10 in DAB stable buffer for thirty minutes followed by a ten-minute wash in running tap water.

Slides were next counterstained with Harris' haematoxylin (see section 2.4) and then dehydrated through graded alcohols (70% ethanol, 90% ethanol, 100% ethanol x2 – 10 dips in each). After clearing the slides with 10 dips in histosol twice, a coverslip was applied using Biomount mounting media.

Each immunoperoxidase staining-run was accompanied by negative controls for both heat-treated and non-heat treated slides. These included diluent alone (1% normal rabbit serum), IgG1 and IgG2a to control for non-specific staining.

2.5.6 Counting Positive Cells for Immunohistochemistry by Light Microscopy.

Prior to counting the candidate was blinded to the origin of the biopsy sample to be examined. All positive cells for immunohistochemistry were counted or scored using a 100 square graticule at x400 magnification. Where positive cells were counted, the area of the whole biopsy sample was calculated and positive cells were expressed per mm^2. Positive cells within glomeruli were counted at x400 magnification. The total number of glomeruli in the biopsy sample was counted and positive cells were expressed per glomerulus.

2.5.7 Semiquantitation of Regulators of Apoptosis by Immunohistochemistry

The counting of immunhistochemically positive cells per mm^2 (as discussed in Section 2.5.6) was possible where the protein of interest was expressed by infiltrating inflammatory cells. For some of the other proteins tested such as Fas and Bcl-x_L, expression was noted mainly in association with tubular epithelial cells. The expression of α-smooth muscle actin was not clearly linked with any specific cell type. (See Figure 5.5 and 5.3) For these proteins a semi-quantitative scoring system was utilised to measure expression.

It was felt by the candidate that measuring Fas and Bcl-x_L positive cells per mm^2 would have been more a reflection of the total number of tubular cells within the section rather than a true measure of protein expression. Furthermore the tubulointerstitial component of each biopsy section varied greatly between patients, making comparisons based upon positive cells per unit area misleading.

The expression of α-smooth muscle actin could not be measured as positive cells per mm^2 as the protein was observed throughout the biopsy samples, not clearly in association with a specific cell type. For this reason semi-quantitative analysis of expression was performed. Other authors have used similar scoring systems for analysis of α-smooth muscle actin expression by immunohistochemistry [166].

Prior to scoring the candidate was blinded to the origin of the biopsy sample to be examined. Expression of the protein of interest was graded from 0 to 3, with 0

representing no positive staining, 1 representing mild staining, 2 representing moderate staining and 3 representing heavy staining.

2.6 Competitive RT-PCR

2.6.1 Utensils and Reagents for RT-PCR

2.6.1.1 Glassware and Plastic Lab Ware

To avoid any contamination with RNAses, all glassware were cleaned thoroughly and baked for 12 hours at 180°C. Re-usable plastic lab ware were thoroughly cleaned and treated for RNAses by soaking them in 0.3% sodium hypochlorite solution for twenty minutes. Only clean and steriie disposable plastic lab-ware was used in the RT-PCR process.

2.6.1.2 Diethylpyrocarbonate (DEPC) Treated Water

DEPC	1ml
RO water	1000ml

The solution was stirred for thirty minutes in a fume cupboard and then autoclaved for thirty minutes.

2.6.1.3 1M Tris Buffer

Tris Base	121.1g
DEPC-treated water	up to 1000ml
Hydrochloric acid, concentrated	as required

Tris base was added to the DEPC-treated water and the solution was titrated to a pH of 7.4 using the hydrochloric acid.

2.6.1.4 Ethylene Diamine-tetra-acetic Acid (EDTA) 500mM

EDTA	93.05g
RO water	500ml
Sodium Hydroxide	as required
Hydrochloric Acid	as required
DEPC	0.5ml

The EDTA was added to 400ml of RO water. In order to dissolve the EDTA the pH of the solution was adjusted to 9.0 with the addition of sodium hydroxide. Once the EDTA was dissolved, the pH was reduced to 8.0 with the addition of hydrochloric acid. The solution was made up to a volume of 500ml with RO water. It was then treated with 0.5ml of DEPC to inactivate any RNAses. The solution was stirred for thirty minutes. Residual DEPC was inactivated by autoclaving the solution for thirty minutes. The solution was stored at room temperature until usage.

2.6.1.5 TE Buffer

1M Tris buffer	5ml
500mM EDTA	1ml
DEPC-treated water	494ml

The solution was stirred for thirty minutes prior to autoclaving for thirty minutes.

2.6.1.6 TAE Buffer x20

Tris base	192.8g
Glacial acetic acid	42.7ml
EDTA 500mM	80ml
DEPC-treated water	up to 2000ml

The reagents were added to the DEPC-treated water and stored at room temperature until use. To make TAE buffer x1, 50 ml of the TAE buffer x20 was added to 950ml of DEPC-treated water.

2.6.2 RNA Extractions

Patients who underwent renal biopsy at The Alfred Hospital with a diagnosis of primary IgA nephropathy were identified by means of the database (see Section 2.8). Only a small proportion of these patients had sufficient excess renal tissue at the time of biopsy to allow freezing and storage of biopsy material for future RNA extraction. At the time of biopsy, written informed consent was received from the patient to use any excess renal tissue for research purposes. Biopsy sections for RNA extraction were snap frozen in liquid nitrogen in a 5ml polypropylene tube and stored at –80°C until extraction was performed.

RNA extractions were performed using the commercially available RNEasy kit (Qiagen, Hilden. Germany). The entire process was performed in a fume cupboard.

β-mercapto-ethanol was added to the Lysis Buffer RLT (10μL/ml of buffer). A tissue sample was then placed in a clean 5ml polypropylene tube with 500μl of the lysis buffer-β-mercapto-ethanol solution. The tissue was then homogenised using a Polytron homogeniser. Following homogenisation, the sample was then centrifuged for three minutes at 3700 revolutions per minute (rpm). If after centrifuging there was still insoluble material visible, homogenisation was repeated followed by a further three minute centrifuge at 3700 rpm.

500μl of 70% ethanol was then added to the sample and mixed gently. 500μl of the sample was then added to an RNEasy spin column and centrifuged for fifteen seconds at 10,000 rpm. The flow-through was discarded. This step was repeated until all the lysate had been spun down.

The sample was then washed with 700μl of Wash Buffer RW1 for fifteen seconds at 10,000rpm in the centrifuge. The sample was then transferred to a clean 2ml tube and washed with 500μl of Wash Buffer RPE for fifteen seconds at 10,000 rpm in the centrifuge. A final wash was performed with 500μl of Wash Buffer RPE for two minutes at 13,000 rpm in the centrifuge.

Using a clean 1.5ml tube, the RNA was eluted from the spin column using 50μl of RNAse free-water and spinning the sample for one minute at 10,000 rpm in the centrifuge. The sample was then stored at –80°C.

The candidate performed twelve extractions; Dr Robyn Langham extracted the rest of the samples.

2.6.3 RNA Quantitation

The total RNA concentration of each sample after extraction was determined using a spectrophotometer (Cary Biol UV-visible Spectrophotometer, Varian Inc, Palo Alto, California. USA). Given that one optical density (O.D) unit at wavelength 260nm is equivalent to 40μg/ml total RNA, samples of RNA were diluted 1:2 to a volume of 6μl in a capillary tube using RNAse free water and the O.D was measured at 260nm spectrophotometrically. Allowing for a capillary cuvette factor of 20 the total RNA concentration of the sample was then calculated by the following equation-

Concentration of total RNA (µg/ml) $= O.D._{260} \times 40 \times 20 \times 2$

$= O.D._{260} \times 1600$

To calculate the purity of the RNA extraction, the O.D. at wavelength 280nm was also measured. Using the equation-

Purity = $O.D._{260}/O.D._{280}$,

an RNA sample was accepted as pure if this ratio fell between 1.8 and 2.0.

Dr Robyn Langham performed RNA quantitation of samples she extracted. The candidate quantified the rest of the samples.

2.6.4 Reverse Transcription (RT)

All reagents used for the RT were from the commercially available GeneAmp RNA PCR kit (Perkin Elmer Life Sciences, Boston, MA. USA). The reaction mixture is shown in Table 2.3.

Reagent	**Volume (µl)**	**Final Concentration**
25 mM magnesium chloride ($MgCl_2$)	4	5mM
10x PCR buffer	2	1x
RNAse free water	2	
Nucleotide tri-phosphates (A,C,T,G)	8	1mM
Rnase inhibitor	1	1 Unit/µl
Murine leukaemic virus reverse transcriptase (MuLV)	1	2.5 Unit/µl
Oligo-thymidilic acid (Oligo dT)	1	2.5mM
RNA	1	
Total	20	

Table 2.3 RT reaction mixture.

The reagents were mixed in a thin walled 500µl eppendorf tube and then 50µl of mineral oil was added to the mixture surface to prevent evaporation of the solution during thermocycling. The RT reaction was carried out in a Peltier Thermal Cycler - 200 using the following protocol –

1. Fifteen minutes at 42°C to allow for reverse transcription.
2. Five minutes at 99°C to inactivate the MuLV enzyme.
3. Five minutes at 4°C for cooling.

2.6.5 Competitive PCR

2.6.5.1 Primer Design

Sequences for the Bcl-2 and Bax primers were designed in the candidate's laboratory by Maria Hernandez and Melissa Egan using the OLIGO™ Primer Analysis software (National Biosciences Inc, Plymouth, MN. USA) based on available gene sequences from Genbank.

All primers were manufactured by Gibco (Invitrogen, Carlsbad, CA. USA). Primer pairs were desalted at the time of construction to improve purity. In order to enable PCR product detection by fluorimager, the upper primers were labelled with fluorescein (fl) at the 5' end. Primer sequences used for the genes Bcl-2 and Bax are shown in Table 2.4

Gene	Primer Sequence
Bcl-2 upper	fl-$^{5'}$CGA CGA CTT CTC CCG CCG CTA CC$^{3'}$
Bcl-2 lower	$^{5'}$CCG CAT GCT GGG GCC GTA CAG TTC C$^{3'}$
Bax upper	fl-$^{5'}$ATG GAC GGG TCC GGG GAG CA$^{3'}$
Bax lower	$^{5'}$GGT GAC CAC TCC CGC CAC AAA GAT$^{3'}$

Table 2.4 Primer sequences for Bcl-2 and Bax.

2.6.5.2 Competitors

Competitive fragments used for RT-PCR of the genes Bcl-2 and Bax were generated and quantified by Maria Hernandez and Melissa Egan in the candidate's laboratory. The competitor was designed to produce a PCR-fragment shorter than the gene of interest and thus allow separation of the two products by electrophoresis.

The concentration of competitor derived was determined using a spectrophotometer and the copy number was calculated from the known base pair size and molecular weight of the competitor fragment. The copy number of the competitors generated is shown in Table 2.5.

Competitor Fragment	Copy Number µg/µl
Bcl-2	4.0354×10^{10}
Bax	1.57×10^{10}

Table 2.5 Copy number of competitors for Bcl-2 and Bax.

2.6.5.3 PCR Reaction For Bcl-2

Primers were reconstituted with TE buffer at a ratio of 1:50 to a concentration of 20 pmol/µl. The competitors were diluted with Rnase free water. All reagents used for the PCR were from the commercially available GeneAmp RNA PCR kit (Perkin Elmer Life Sciences, Boston, MA. USA). The final, optimised reaction mixtures for Bcl-2 and Bax are shown in Table 2.6.

Reagent	Bcl-2 Reaction Volume (µl)	Bax Reaction Volume (µl)
25 mM $MgCl_2$	1.0	1.5
10 x PCR buffer	2.25	2.25
Rnase-free water	16.0	15.5
AmpliTaq gold	0.25	0.25
Upper primer	1.0	1.0
Lower primer	1.0	1.0
CDNA	2.5	2.5
Competitor	1	1
Total	**25**	**25**

Table 2.6 PCR reaction mixture.

The reagents were mixed in a thin walled 500µl eppendorf tube and then 50µl of mineral oil was added to the mixture surface to prevent evaporation of the solution during thermocycling.

The PCR reaction was carried out in a Peltier Thermal Cycler - 200 using the following protocol –

1. Twelve minutes at 94°C to activate the AmpliTaq gold enzyme.

2. Forty seconds at 94°C to allow denaturing of the double-stranded DNA.

3. One minute at primer-specific temperature (Table 2.7) to allow primer annealing to single-stranded DNA.

4. One minute and thirty seconds at 72°C for primer extension.

5. Steps 2–4 were repeated for a primer-specific number of cycles. (Table 2.7)

6. Five minutes at 72°C for final extension.

7. Minimum of thirty minutes at 4°C to allow cooling of solution and inactivation of AmpliTaq gold enzyme.

Competitor	Annealing Temperature	Optimised Cycle Number
Bcl-2	65°C	40
Bax	67°C	40

Table 2.7 Annealing temperatures and cycle number for Bcl-2 and Bax PCR.

2.6.6 Agarose Gel Preparation and PCR Electrophoresis

All PCR products were separated by agarose gel electrophoresis and then fluorometrically scanned. A 2% agarose gel was made by adding 2g of agarose to 50ml of TAE x1 buffer in a clean, conical flask. The solution and flask were weighed and then an extra 20ml of RO water was added to the mixture to allow for evaporation on boiling. The solution was boiled for approximately two minutes in the microwave until the agarose was completely dissolved. The solution was then weighed again and RO water was added if necessary to restore the solution to its original volume (and 2% concentration).

The solution was then cooled slightly by running the flask under cold, tap water and then poured into a horizontal gel mould with a 12-well comb. It was then left to cool and solidify for at least 30 minutes at room temperature prior to use. The gel was placed in an electrophoresis tank and immersed in approximately 250ml of x1 TAE buffer prior to receiving the PCR product.

PCR samples were prepared for electrophoresis by adding 9µl of the PCR product to 1µl of Promega Blue/Orange x6 Loading Dye in a 0.5ml sterile eppendorf tube. The samples were centrifuged briefly and then loaded into the gel wells in the electrophoresis tank. Samples were electrophoresed at a constant 100 V for 40 minutes. The gel was then fluorometrically scanned using a Fluoroimager™ 575. Images were scanned at 650 volts initially, and then adjusted to ensure an optical density was achieved for each band where possible. (See Figure 2.1 for example) The output from each band (as volume of pixel generated) was then measured using ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA. USA).

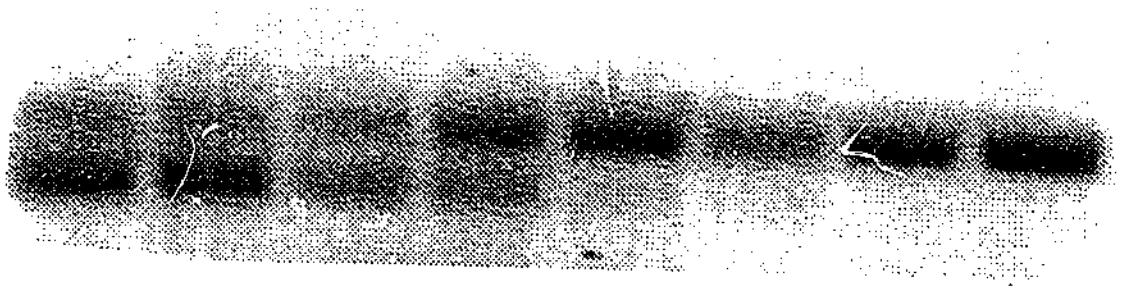


Figure 2.1 An example of a gel fluorometrically scanned using the Fluoroimager™ 575.

2.6.7 Statistical Analysis for RT-PCR

All statistical analysis for the RT-PCR was performed using SPSS for Windows. The copy number of the gene of interest in each sample was determined using the point of equivalence with the known competitor copy number; that is the point where the ratio of known competitor copy number over unknown target copy number is equal to one. The ratio of the log of the known competitor copy number over the log of the unknown target copy number was measured for each sample using five different concentrations of competitor copy number. (Table 2.8)

Band number	Band output (pixel volume)	Log (competitor copy number)	Log (ratio competitor band output /target band output)
1	41698	5.004	-1.023
2	55459	4.402	-0.732
3	131848	3.8	-0.09
4	192410	3.198	0.524
5	260629	2.596	1.0499
6	440414		
7	299668		
8	162327		
9	57472		
10	23233		

Table 2.8 Data for determination of equivalence point.

A graph was then created to demonstrate the relationship between the log (ratio) versus log (competitor copy number). The concentrations of competitor were titrated for each sample to ensure the graph crossed the X-axis (i.e. the equivalence point where log = 0). (Figure 2.2) Statistical analysis of the graph using linear regression accurately determined the equivalence point. The regression analysis was considered acceptable if the R^2 value > 90 and $p < 0.05$. Any PCR sample that did not meet these criteria was repeated.

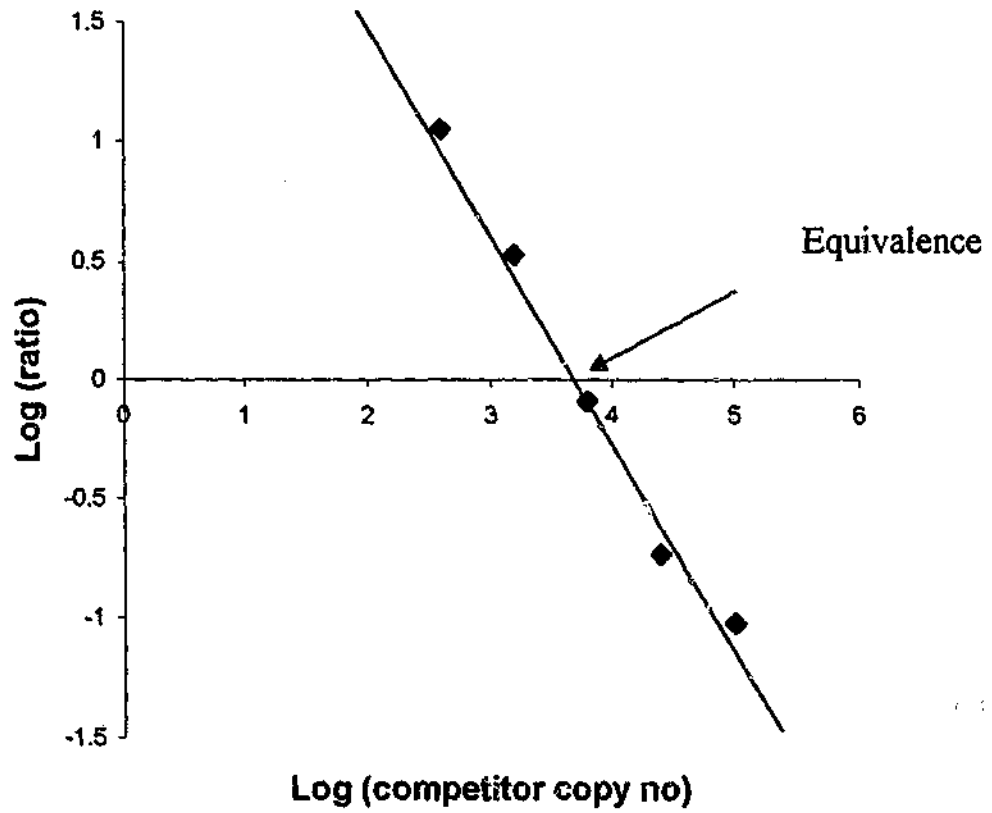


Figure 2.2 Graph for determining equivalence point.

The copy number of the gene of interest for each sample was determined from the equivalence point based on the known copy number of the competitor. The final copy number was expressed as copies per ng total RNA as determined for each sample (see Section 2.6.3).

2.6.8 Optimising RT-PCR

In order to identify the optimal conditions for amplification of PCR products, experiments were performed that examined the effect of magnesium chloride concentration, primer concentration and cycle number on PCR output. These were utilised to establish the ideal conditions for optimal PCR yield and efficiency. The following are the protocols used and results obtained for the optimisation of Bax. Similar protocols were utilised for optimisation of Bcl-2. The results of the optimisation experimentation for Bcl-2 are not shown. However the final optimized reaction mixtures derived from these experiments are shown in Table 2.6.

2.6.8.1 Optimisation of Magnesium Chloride Concentration

After preparing a stock solution of reverse transcription mix four PCR reactions were prepared, each with an increasing concentration of $MgCl_2$. The four concentrations of $MgCl_2$ utilised were 0.5mM, 1.0mM, 1.5mM and 2.0mM. The formulae for the four PCR reactions are listed in Table 2.9.

Reagent	PCR 1 Volume (µl)	PCR 2 Volume (µl)	PCR 3 Volume (µl)	PCR 4 Volume (µl)
25 mM $MgCl_2$	0	0.5	1.0	1.5
10 x PCR buffer	2.25	2.25	2.25	2.25
Rnase-free water	17.4	16.9	16.4	15.9
AmpliTaq gold	0.25	0.25	0.25	0.25
Upper primer	0.8	0.8	0.8	0.8
Lower primer	0.8	0.8	0.8	0.8
CDNA	2.5	2.5	2.5	2.5
Competitor	1.0	1.0	1.0	1.0
Total	**25**	**25**	**25**	**25**

Table 2.9 Formulae used for $MgCl_2$ optimisation for Bax.

The output from the resultant gel from the four PCR reactions was expressed as a ratio of competitor band output versus target band output for each reaction. The ratios were then graphed against the $MgCl_2$ concentration. The concentration of $MgCl_2$ that yielded the highest output ratio was selected for use in future PCR reactions for Bax. In this case a $MgCl_2$ concentration of 2.0mM was selected. (See Figure 2.3)

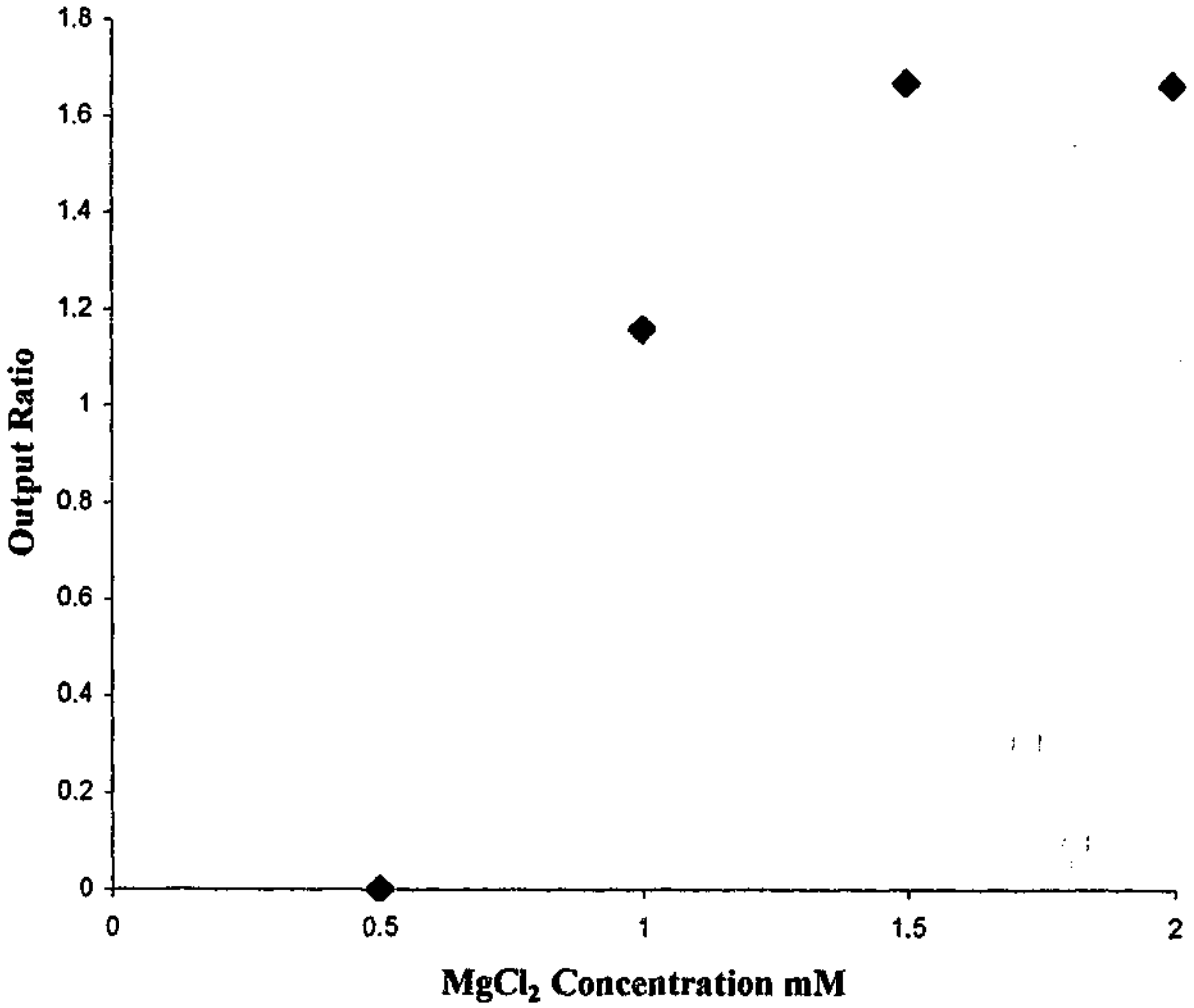


Figure 2.3 Graph of PCR output ratios versus $MgCl_2$ concentration.

2.6.8.2 Optimisation of Primer Concentration

After preparing a stock solution of reverse transcription mix four PCR reactions were then prepared, each with a varying concentration of primer. The concentrations of primer tested included: 0.4 pmol/µl, 0.64 pmol/µl, 0.8 pmol/µl and 1.0 pmol/µl. The PCR formulae for each reaction are shown in Table 2.10.

Reagent	PCR 1 Volume (µl)	PCR 2 Volume (µl)	PCR 3 Volume (µl)	PCR 4 Volume (µl)
25 mM $MgCl_2$	1.5	1.5	1.5	1.5
10 x PCR buffer	2.25	2.25	2.25	2.25
Rnase-free water	16.5	15.9	15.5	15.0
AmpliTaq gold	0.25	0.25	0.25	0.25
Upper primer	0.5	0.8	1.0	1.25
Lower primer	0.5	0.8	1.0	1.25
CDNA	2.5	2.5	2.5	2.5
Competitor	1.0	1.0	1.0	1.0
Total	**25**	**25**	**25**	**25**

Table 2.10 Formulae used for optimisation of primer concentration.

The output from the resultant gel from the four PCR reactions was expressed as a ratio of competitor band output versus target band output for each reaction. The ratios were then graphed against the primer concentration. The concentration of primer that yielded the highest output ratio was selected for use in future PCR reactions for Bax. In this case a primer concentration of 0.8 pmol/µl was selected, corresponding to a primer volume of 1.0µl (see Figure 2.4).

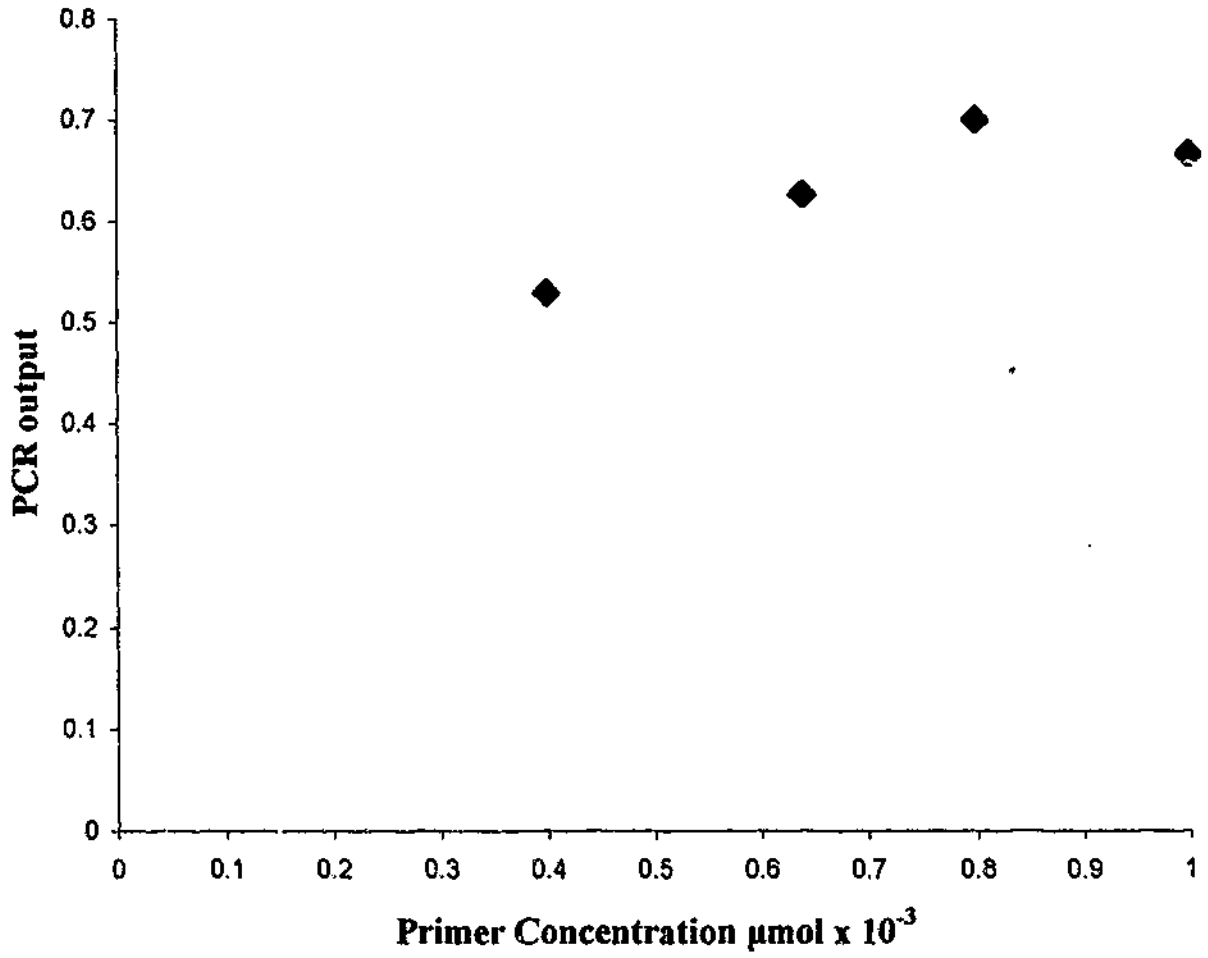


Figure 2.4 Graph of PCR output ratios versus primer concentration.

2.6.8.3 Optimisation of Cycle number

In order to optimise the PCR cycle number, ten PCR solutions were prepared using a stock solution of reverse transcription. The solutions were prepared using the already optimised $MgCl_2$ and primer concentrations. Each solution was run on the Peltier Thermal Cycler - 200 for an increasing number of programmed cycles, starting at 35 and increasing to 46 cycles.

The output from the resultant gel from the ten PCR reactions was expressed as a ratio of competitor band output versus target band output for each reaction. The ratios were then graphed against the cycle number (Figure 2.5). It was clear from this graph that increases in cycle number above 40 did not significantly improve the PCR yield. Therefore PCR reactions for Bax were run through 40 PCR cycles only.

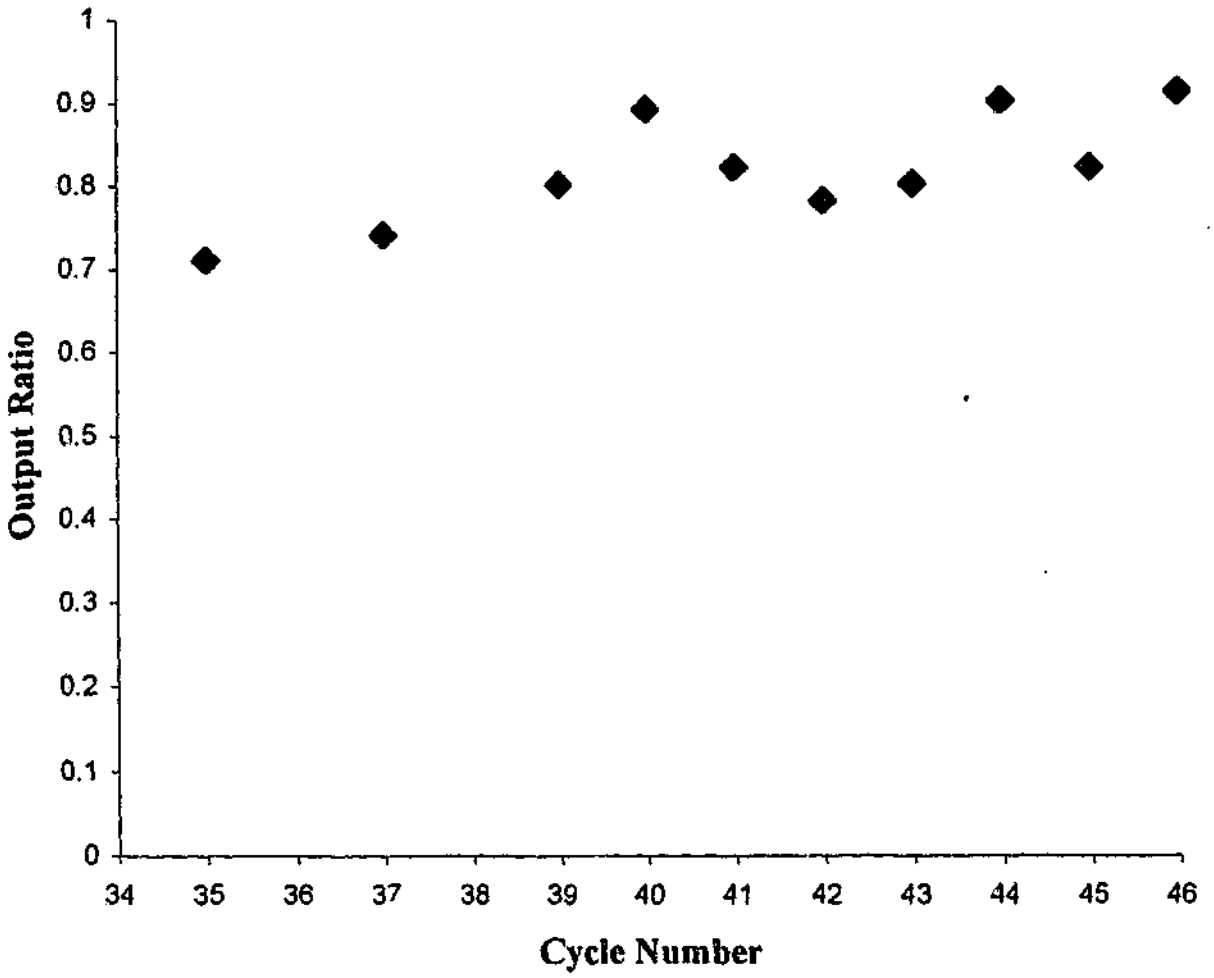


Figure 2.5 Graph of PCR output ratios versus cycle number.

2.6.9 Construction Of Standard Curves For Bax and Bcl-2

Once the PCR reactions for both Bax and Bcl-2 were optimised for cycle number, primer and $MgCl_2$ concentration, standard curves were constructed from three dilutions of RNA. This confirmed the accuracy and reliability of the individual PCR reactions. Three concentrations of RNA were used in dilutions of 1:2, 1:1 and 2:1. A coefficient of variation of < 25% for the three dilution reactions was deemed acceptable for the PCR protocol to be reliably utilised.

Figure 2.6 and 2.7 show the results of the standard curve validation experiments with associated coefficients of variations for Bax and Bcl-2 respectively.

Figure 2.6 Validation experiment for Bax RT-PCR. Three standard curves correspond to 1:2, 1:1 and 2:1 dilutions.

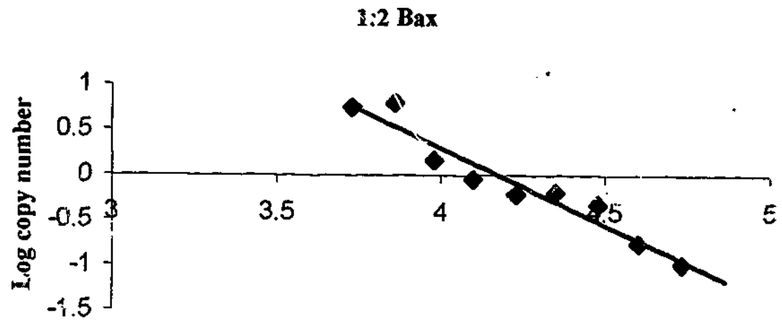
Results for each equivalence point were-

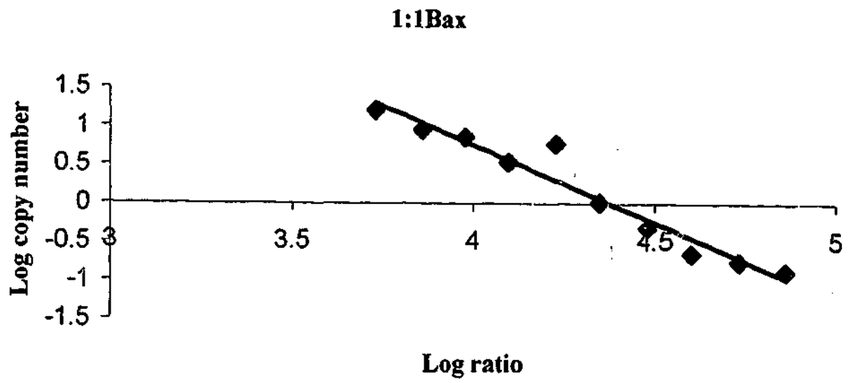
Dilution	Equivalence Point
1:2	12809
1:1	21352
2:1	31600

Coefficient of variation was calculated as follows -

(Standard deviation/mean) x 100 = 2462/10461 x 100

= **23%**





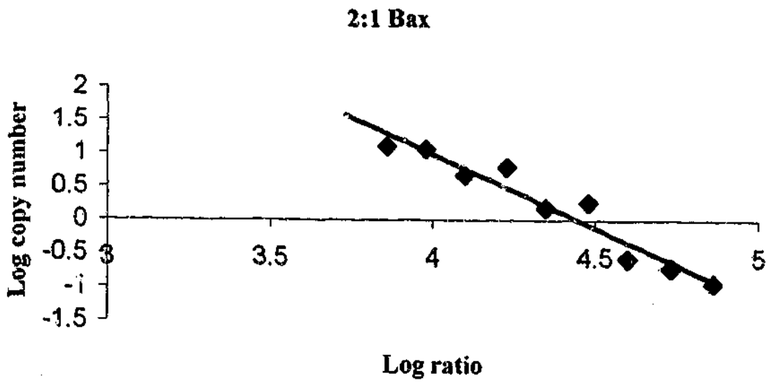


Figure 2.7 Validation experiment for Bcl-2 RT-PCR. Three standard curves correspond to 1:2, 1:1 and 2:1 dilutions.

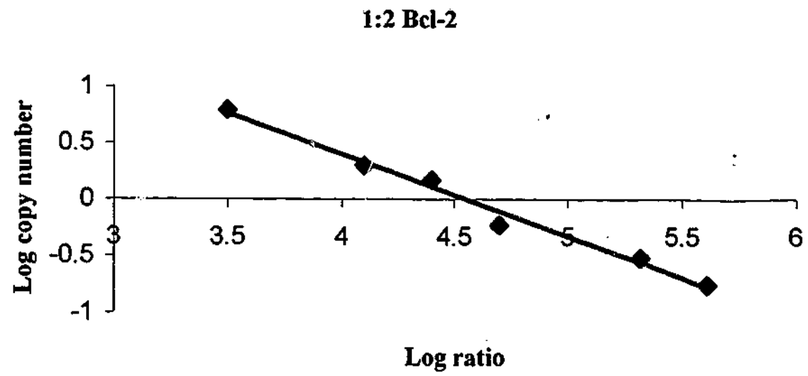
Results for each equivalence point were-

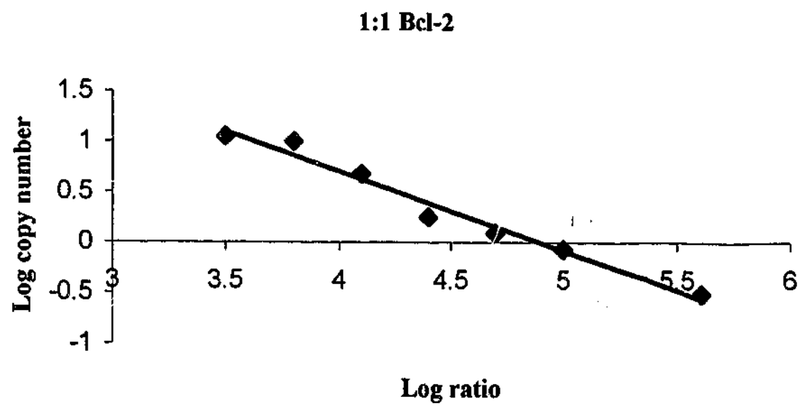
Dilution	Equivalence Point
1:2	35576
1:1	70275
2:1	120357

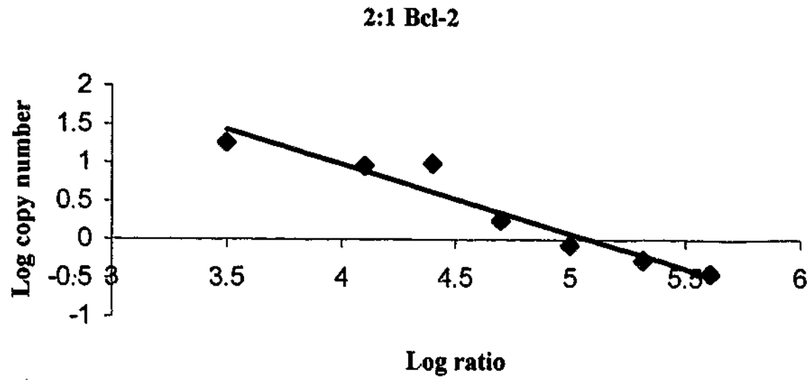
Coefficient of variation was calculated as follows -

(Standard deviation/mean) x 100 = 3049/33601 x 100

= **9%**







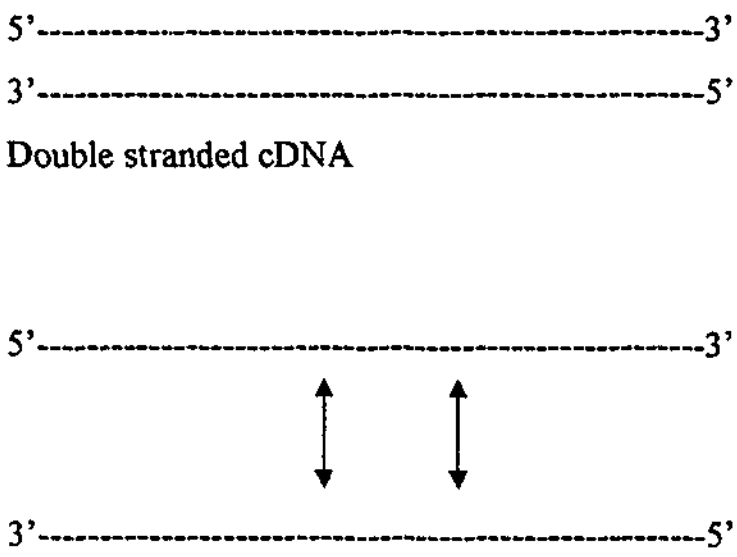
2.7 Real-Time PCR

2.7.1 Introduction

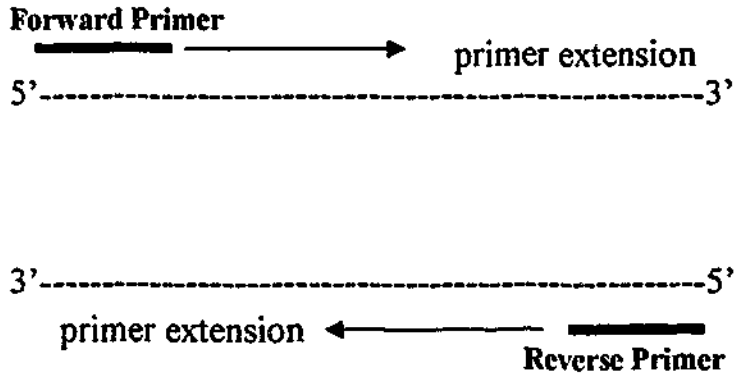
Real-time PCR relies on relatively new technology that allows quantification of gene expression in human and animal tissues. The process is based around the principles of reverse transcription of RNA and the polymerase chain reaction to amplify specific target gene sequences. As with competitive PCR, the process takes place in two parts: synthesis of cDNA from total RNA and amplification of cDNA with fluorogenic detection and quantification of the PCR product. However unlike competitive PCR, the amplification of the target gene sequence and its relative quantification take place at the same time, within the one instrument.

In the presence of an optimised concentration of PCR buffer, magnesium chloride and DNA polymerase, gene specific primers and probe are added to cDNA reverse transcribed from extracted tissue RNA. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Boston, MA. USA) the mixture is then heated and cooled through a range of specific temperatures. This allows first separation of double stranded cDNA followed by the annealing of the gene specific primers to target gene sequences in the single stranded cDNA. Finally a new strand of cDNA is synthesised by extension of the primers, to reform the double stranded cDNA. The separation/annealing/extension cycle is repeated up to 40 times to ensure many thousands of copies of the gene of interest. (Figure 2.8)

Figure 2.8 The Polymerase Chain Reaction.



Step 2. Gene specific forward and reverse primers anneal to separated DNA strands at 60°C.



Step 3. Extension of primers reform double stranded DNA; note sequence of interest is now doubled in quantity.

Step 4. Solution is heated to 95°C again, to allow separation of double stranded DNA and steps 2 and 3 are repeated to form four times the amount of the sequence of interest.

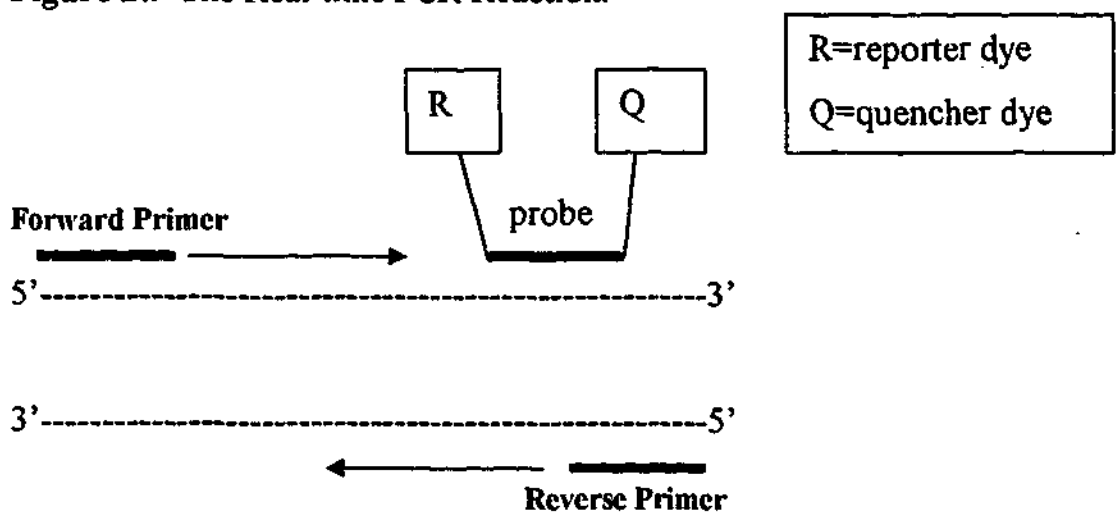
The cycle is repeated up to forty times to ensure multiple copies of the gene of interest are manufactured.

As the above reaction occurs the fluorescent output from the solution increases in direct relationship to the number of copies manufactured of the gene of interest. This occurs as a result of the specially designed probe present in the solution that contains a reporter dye at its 5'-end and a quencher dye at its 3'-end. In its intact state, the probe emits no fluorescence as the quencher dye suppresses the reporter dye output due their close proximity.

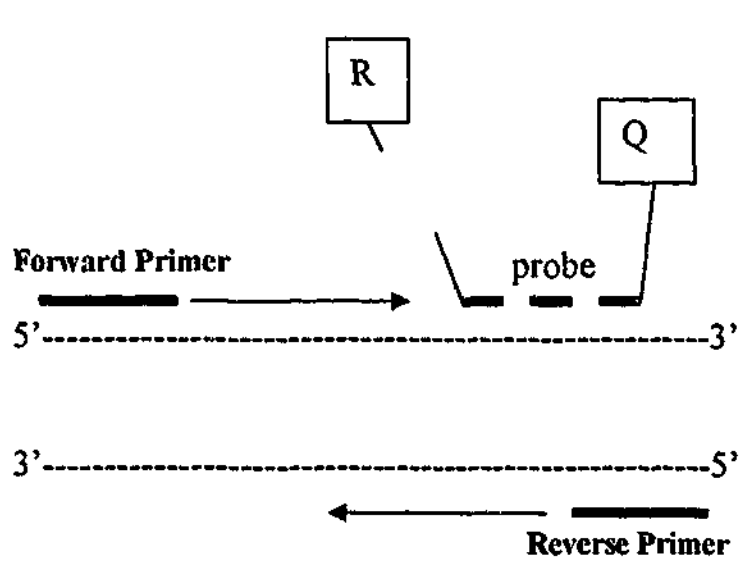
The probe is specifically designed to anneal to the gene of interest between the forward and reverse primer sites. The 3' end of the probe is blocked to prevent extension of the probe during PCR. Once annealed to the cDNA template the AmpliTaq Gold® DNA polymerase cleaves the probe between the reporter and quencher dye. The cleavage only occurs if the probe is hybridised to the target gene sequence. The fragments of the probe are then displaced from the template and polymerisation of the strand continues. With cleavage of the probe, the reporter dye and quencher dye separate allowing the reporter dye to emit its fluorescent signal. The specificity of the

probe to the gene of interest means that cleavage of the probe and thus release of the fluorescent signal only occurs in the presence of a copy of the gene of interest. In this way non-specific amplification is not detected. (Figure 2.9)

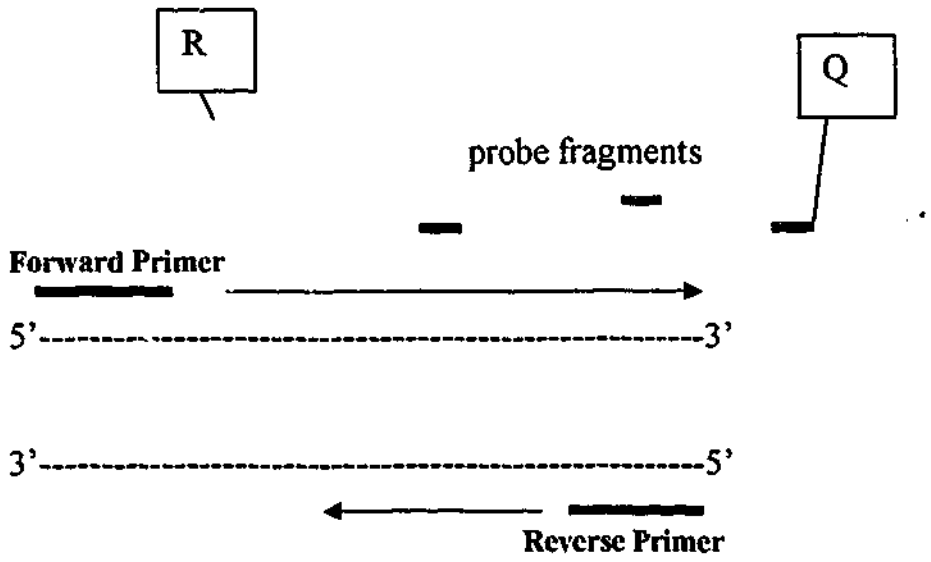
Figure 2.9 The Real-time PCR Reaction.



Step 1. Probe anneals to the gene of interest between the forward and reverse primer sites, fluorescence is suppressed by the proximity of the reporter and quencher dyes.



Step 2. With heating of the solution to 95°C, the AmpliTaq Gold® DNA polymerase is activated and cleaves the probe between the reporter and quencher dyes, only if it hybridises to the gene of interest.



Step 3. The fragmented probe detaches from the cDNA and further extension of the strand continues. With separation of the reporter and quencher dye, the reporter dye emits a fluorescent signal that is measured by the instrument.

2.7.2 RNA Extractions

RNA extraction for the real-time PCR was performed as described for competitive PCR (see Section 2.6.2).

2.7.3 Reverse Transcription (RT) for Real Time PCR

All reagents used for the RT were from the commercially available GeneAmp RNA PCR kit (Perkin Elmer Life Sciences, Boston, MA. USA). The reaction mixture is shown in Table 2.11.

Reagent	**Volume (µl)**	**Concentration**
25 mM magnesium chloride ($MgCl_2$)	4	5mM
10x PCR buffer	2	1x
RNAse free water	2	
Nucleotide tri-phosphates (A,C,T,G)	8	1mM
Rnase inhibitor	1	1 Unit/µl
Murine leukaemic virus reverse transcriptase (MuLV)	1	2.5 Unit/µl
Random hexamer	1	2.5mM
RNA	1	
Total	**20**	

Table 2.11 RT reaction mixture for real-time PCR.

Random hexamers were utilized for the real-time reverse transcription rather than oligo-thymidilic acid (Oligo dT) to ensure that total RNA was transcribed and not just messenger RNA. This was necessary as the 18S component of ribosomal RNA was to be used as an endogenous control. The reagents were mixed in a thin walled 500µl eppendorf tube and then 50µl of mineral oil was added to the mixture surface to prevent evaporation of the solution during thermocycling. The RT reaction was carried out in a Peltier Thermal Cycler - 200 using the following protocol –

1. Fifteen minutes at 42°C to allow for reverse transcription.
2. Five minutes at 99°C to inactivate the MuLV enzyme.
3. Five minutes at 4°C for cooling.

2.7.4 Real-Time PCR Reactions

2.7.4.1 Reagents for Real-Time PCR

Real-time PCR was performed using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Boston, MA. USA). Pre-developed assay reagents TaqMan® (PDARs) were purchased for the genes Fas and Fas-ligand from Applied Biosystems. These reagents are optimised to work with the TaqMan® Universal PCR Master Mix (Applied Biosystems, Boston, MA). The endogenous control 18S ribosomal RNA (Applied Biosystems, Boston, MA. USA) was used for all real-time PCR reactions. Initial attempts to establish a standard curve for both genes of interest were made using Raji® total human RNA suppled by Applied Biosystems.

2.7.4.2 Thermal Cycling

Thermal cycling was performed by the ABI PRISM® 7700 Sequence Detection System. The following standardised protocol was utilised for all real time PCR reactions.

Step 1. Two minutes at 50°C to activate AmpErase® uracil-N-glycosylase, an enzyme that inhibits genomic DNA replication.

Step 2. Ten minutes at 95°C for pre-activation of AmpliTaq Gold ® DNA polymerase.

Step 3. Fifteen seconds at 95°C for separation of double stranded cDNA.

Step 4. One minute at 60°C for annealing and extension.

Step 3 and 4 repeated thirty nine times.

2.7.4.3 Data Output and Analysis

An example of the data output from the ABI PRISM® 7700 Sequence Detector is shown in Figure 2.10. Analysis of the data generated was performed as outlined in the SDS Compendium 7700-Version 4.0 (Applied Biosystems, 2001). In brief this involved excluding outlying wells, setting the baseline and threshold bars.

Outlying wells were wells that failed to amplify in log-linear fashion. Viewing output in the linear amplification plot best identified these outlying wells. Any well without at least 3 points in the log amplification phase was deleted.

The baseline was used to calculate the background output from un-reacted probe. From this baseline the threshold was automatically calculated. Therefore prior to

determining the threshold value, the baseline had to be set. Baseline was set 1-2 cycles before the reporter dye signal began to increase. If amplification was observed to occur after cycle 15 the baseline was not altered from its default setting of 3 to 15.

Threshold values were set manually within the exponential phase of the logarithmic scale amplification plot. This corresponded to the approximate mid-point of the linear component of the amplification curve. Once the threshold was established for the entire PCR run, Ct values were produced by the Sequence Detector for each individual PCR reaction. The Ct value for an individual reaction corresponds to the point at which linear replication of the target sequence takes place and is inversely proportional to the amount of target cDNA present in the sample. Target sequences abundant in the cDNA sample amplify at an earlier PCR cycle than those present in lesser amounts and thus have a corresponding low Ct value.

The Ct value for each individual reaction was then utilized to determine the relative amount of a target gene and/or a control gene within a specific test sample. The methodology used in converting a given Ct value into a relative PCR product amount will be discussed in detail in Chapter Seven.

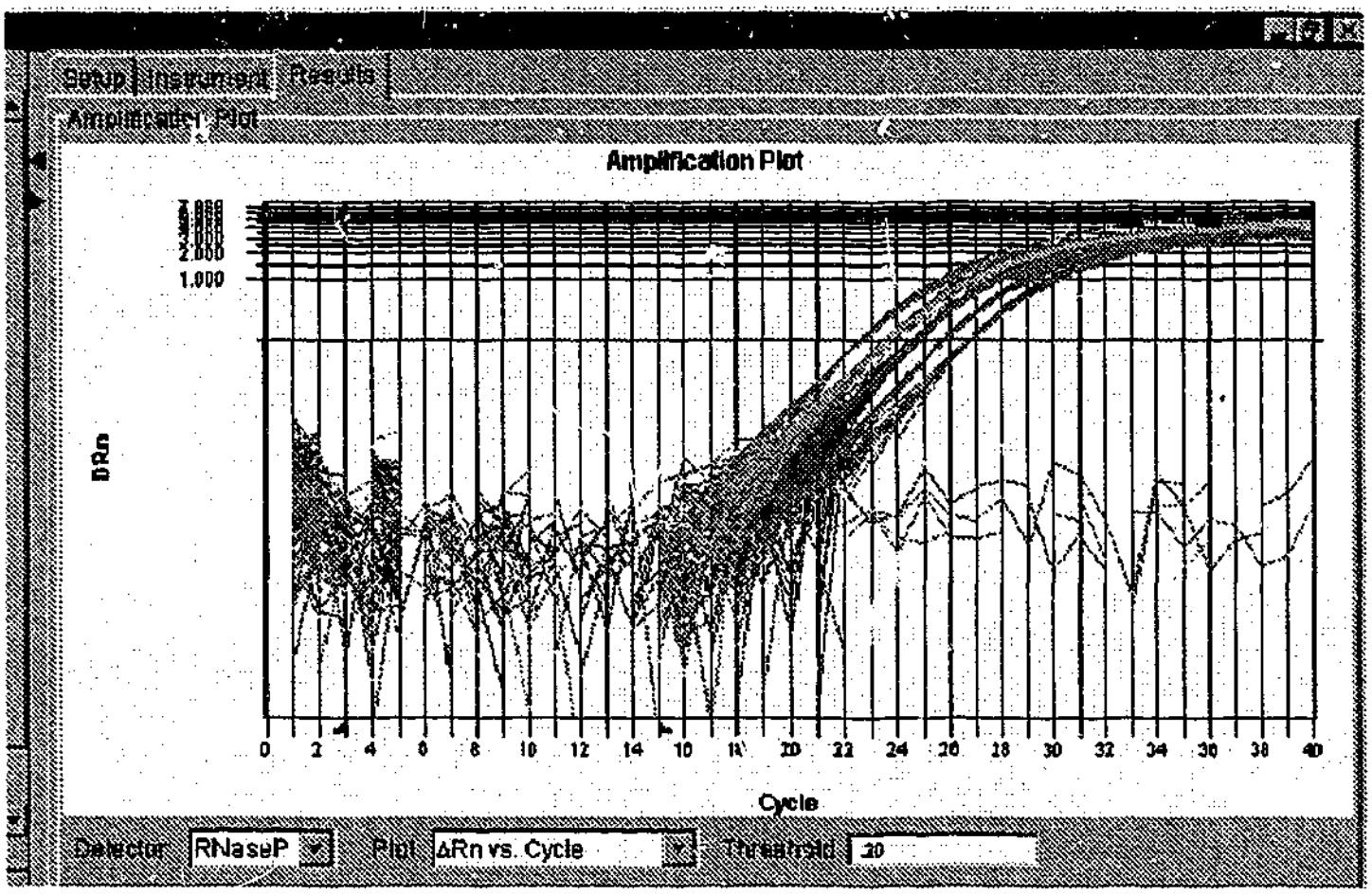


Figure 2.10 An example of data output from the ABI PRISM® 7700 Sequence Detector. (from [167])

2.7.4.4 Separate-Well Real-Time PCR Reactions

Initially all reactions were performed in separate wells to familiarise the candidate with the technology. The analysis of the target gene Fas was performed using this method. The PCR reactions for the gene of interest and the endogenous controls were run in separate wells on the same plate. All reactions were 20µl in total volume and all samples were run in triplicate. All sample runs were performed with a no template control in triplicate that contained no RNA as a negative control. The reaction mixture for separate-well PCR is shown in Table 2.12.

Reagent	**Volume (µl)**
RNAse free water	6.5
TaqMan® PDAR probe and primer (gene of interest **or** endogenous control)	1.0
TaqMan® Universal PCR master mix	10.0
RNA	2.5
Total	**20**

Table 2.12 Reaction mixture for separate-well real-time PCR.

2.7.4.5 Multiplex Real-Time PCR Reactions

The ABI PRISM® 7700 Sequence Detection System is able to distinguish different reporter dyes within the same well. This allows both target gene and endogenous control to be amplified within the same well provided they use different reporter dyes. The 18S ribosomal RNA endogenous control uses VIC reporter dye and Fas-ligand uses FAM thus enabling a multiplex system to be used for this target gene.

The advantage of using a multiplex system for real-time PCR is that it reduces reagent and RNA requirements and is somewhat quicker to prepare. As well as this, the sample RNA is added to a single well for analysis of both target and control gene expression thus eliminating an important source of pipetting error that exists in the separate well system. The reaction mixture for multiplex real-time PCR is shown in Table 2.13.

Reagent	Volume (µl)
RNAse free water	5.5
TaqMan® PDAR probe and primer for gene of interest	1.0
TaqMan® PDAR probe and primer for endogenous control	1.0
TaqMan® Universal PCR master mix	10
RNA	2.5
Total	**20**

Table 2.13 Reaction mixture for multiplex real-time PCR.

2.8 IgA Database

2.8.1. Inclusions and Exclusions

A database of all patients with a diagnosis of primary IgA nephropathy seen at The Alfred Hospital, Melbourne was constructed. All renal biopsies performed at The Alfred Hospital between 1975 and 2002 were surveyed and patients with a diagnosis of IgA nephropathy were identified.

As the aim of the database was to characterise the natural history of *primary* IgA nephropathy at this institution, any patient with presumed secondary IgA nephropathy or HSP was excluded from analysis. As well as this, patients with recurrent primary IgA nephropathy in a renal transplant were also excluded from analysis. This was because the natural history of *recurrent* IgA nephropathy is quite different from primary IgA nephropathy as it may be influenced by other factors present within the transplanted kidney, such as chronic rejection and/or cyclosporine nephrotoxicity [168].

Any patient with primary IgA nephropathy as well as a second, unrelated diagnosis on renal biopsy such as diabetic nephropathy was also excluded from analysis for similar reasons. Patients with primary IgA nephropathy and associated hypertensive

nephrosclerosis or secondary focal and segmental glomerulosclerosis were included in the analysis as the principal lesion was assumed to be IgA nephropathy.

The database was constructed as a tool for analysis of cellular apoptosis in progressive versus non-progressive IgA nephropathy. As the database was a complete representation of all patients with IgA nephropathy seen at The Alfred Hospital over the last 27 years, it was also used as a tool to try and identify those factors both clinical and histological which were linked to disease progression within this patient population.

2.8.2 Patient Consent.

At the time of renal biopsy, all patients gave written, informed consent for results and data to be used for the purpose of medical research. Ethics approval was granted by The Alfred Hospital Ethics Committee for the construction of the database and subsequent tissue analysis. However, as the database was to be utilised as an aid to further studies being performed in the laboratory, it was decided that additional consent be sought from the patients specific to the further studies that were to be carried out. This consent included patient permission for enrolment in the database and permission for further genetic analysis to be performed on serum collected from the patients.

2.8.3 Baseline Parameters and Follow-up

Once identified, patient's histories were examined to establish their age at the time of biopsy, sex and the date of biopsy. For the purposes of establishing a minimum follow-up period, it was felt that date of biopsy was a more reliable starting point than date of onset of symptoms given the retrospective nature of the analysis. In general these two dates were often only separated by several months, but for many patients this could not be reliably established.

Renal parameters at biopsy were obtained, including serum creatinine concentration (Cr), creatinine clearance (CrCl) and 24-hour protein excretion. Mid stream urine (MSU) at the time of biopsy was obtained to document the degree of glomerular haematuria. This was graded as normal (<10 red blood cells (RBC) $x10^6$/L), moderate (10-100 RBC$x10^6$/L) or severe (>1000 RBC$x10^6$/L). Any patient with a history of macroscopic haematuria was graded as "severe" regardless of degree of haematuria at the time of biopsy.

Serum IgA at the time of biopsy was obtained and recorded as normal (range 0.8-4.5g/L) or elevated (>4.5g/L). This was the only parameter that was not consistently measured in the patient population and was available in only 55% of patients.

An attempt was made to establish the average blood pressures recorded around the time of renal biopsy. Again, given the retrospective nature of the analysis, this proved difficult and therefore an approximate grading system was used based on recorded blood pressures. Blood pressure was defined as "normal" if it was documented to be consistently below 130/90 mmHg, "moderate" if it ranged between 130-180/90-100 mmHg and "severe" if it was consistently measured at greater than 180/100 mmHg or was associated with symptoms or signs consistent with malignant hypertension.

The patients being followed-up at The Alfred Hospital were identified. Current renal parameters were obtained from the patients' medical records. These included CrCl and 24-hour protein excretion as well as a mid-stream urine collection. Those patients in whom insufficient data was available from the medical record as well as patients that were not being followed-up at the Alfred Hospital were contacted by the candidate. It was arranged for the patient to have a 24-hour urine collection to measure CrCl and protein excretion. A mid-stream urine was also collected to measure the degree of haematuria. Those patients lost to follow-up who were contacted by the candidate were encouraged to attend The Alfred Hospital for appropriate follow-up.

2.8.4 Renal Biopsies

Renal biopsy reports were obtained for each patient. The majority of patients were biopsied at The Alfred Hospital but for some patients other institutions had to be contacted for the reports. The biopsies were graded for proliferative injury and sclerosing injury based on the pathology report. If the report was insufficient in detail the original biopsy slides were examined by the candidate often with the help of a pathologist from the Department of Anatomical Pathology and graded directly from the slides.

To provide some reassurance on the accuracy of biopsy scoring performed by the candidate, it would have been ideal to have the biopsies scored by a second observer blinded to the patient details. Unfortunately due the large amount of biopsy material examined this was not practical. Despite this a small number of biopsies *were* scored by

both a pathologist and the candidate to ensure there was agreement on the definition of various biopsy parameters between two independent observers. Once these parameters were defined they were consistently applied to all the biopsy specimens examined.

The biopsy scoring system used in the thesis was devised by the candidate in an effort to separate glomerular proliferative changes from glomerular sclerosing changes. A similar system was utilised by Andreoli et al [169] in a study on the effects of treatment on biopsy parameters in a paediatric population with IgA nephropathy. This system of scoring focuses mostly on glomerular injury, with a lesser weighting for tubulointerstitial injury. Other authors such as Haas et al [46], have combined sclerosing and proliferative injury into a single score to express biopsy injury patterns. A further methodology utilised to score IgA renal biopsies involves separate scoring for glomerular, interstitial and vascular injury [170].

As the biopsy database was to be used as a tool for exploring apoptosis in IgA nephropathy it was felt by the candidate that a scoring system that distinguished between proliferative injury and sclerosing injury would be most useful for the purposes of this study. Whilst this style of scoring may underplay the role of tubulointerstitial injury in the progression of disease, it allowed the candidate to focus on the processes that may be involved in the resolution of glomerular inflammation and the development of glomerulosclerosis.

The biopsy slides were scored for proliferative changes. These included; degree of mesangial proliferation, presence of focal necrotizing lesions, presence of crescents and degree of interstitial infiltrate (see Table 2.14 for scoring details).

The slides were next scored for sclerosing changes. These included; percentage of global sclerosis, percentage of segmental sclerosis, degree of mesangial thickening, degree of vascular changes, degree of interstitial fibrosis and atrophy (see Table 2.15 for scoring details).

Proliferative Index	Degree	Score
Mesangial proliferation	Nil	0
	Mild	1
	Moderate	2
	Severe	3
Focal necrotizing lesions	Absent	0
	Present	2
Crescents	Nil	0
	<10% of glomeruli	1
	10-50% of glomeruli	3
	>50% of glomeruli	5
Interstitial infiltrate	Nil	0
	Mild	1
	Moderate	2
		Total score /12

Table 2.14 Proliferative Index scoring system for renal biopsies.

Sclerosis Index	Degree	Score
Global sclerosis	0-10% of glomeruli	0
	10-30% of glomeruli	1
	30-60% of glomeruli	2
	>60% of glomeruli	3
Segmental sclerosis	Nil	0
	0-30% of glomeruli	1
	30-60% of glomeruli	2
	>60% of glomeruli	3
Mesangial thickening	Nil	0
	Mild	1
	Moderate	2
Vascular changes	Nil	0
	Mild	1
	Moderate	2
Interstitial fibrosis / atrophy	Nil	0
	Mild	1
	Moderate	2
		Total /12

Table 2.15 Sclerosing Index scoring system for renal biopsies.

The proliferative index and sclerosis index were then combined to give an overall renal injury score. The scorings system was designed by the candidate to ensure that within the overall renal injury score, proliferative changes were given equal weighting to sclerosing changes. Although this is somewhat artificial, it was done in an attempt to establish whether proliferative change or sclerosing change on biopsy independently contributed to disease progression rather than the degree of overall renal injury.

The intensity of immunostaining on the renal biopsy for IgA was also noted and graded as mild, moderate or heavy in an attempt to see whether this was significantly associated with progression of disease. The presence or absence of IgG staining within glomeruli was also noted.

2.8.5 Progression Versus Non-progression

Patients were defined as "progressors" or "non-progressors" based on their follow-up renal function. Given the slow rate of progression that can be observed in IgA nephropathy it was decided that a minimum of two years follow up with stable renal function was required for a patient to be defined as a non-progressor. Creatinine clearance (CrCl) was measured with a 24-hour urine collection. Where this was not available, an individual's CrCl was determined using the Cockcroft and Gault formula [171] based on current serum creatinine, the patient's age, sex and weight. The majority of patients provided a 24-hour urine collection, which was felt to be a more accurate measure of renal function.

A patient was defined as a non-progressor on the basis of a stable glomerular filtration rate (GFR) at last follow-up. Increasing proteinuria, ongoing haematuria or significant hypertension were not considered markers of progressive renal injury. If a patient had significant renal impairment at presentation, but renal function was stable at last follow-up (minimum of two years) they were defined as non-progressors. This was the case in only a few individuals.

From what has been discussed with regards to the progression of IgA nephropathy, it would have been ideal to collect a minimum of 5 years follow-up on patients before classifying them as non-progressors. Some authors would argue that 10 years follow-up would be a minimum. Unfortunately due to the shortage of renal biopsy specimens available to the candidate particularly for RNA extraction, this would have significantly restricted the patient numbers available for analysis. At two years follow-up with stable renal function it can at least be said that progression of disease is less likely although not inevitable. It should be noted however, that the *mean* length of follow-up for patients was in fact 5 years.

Progressive IgA nephropathy was defined as a significant decline in renal function from baseline at the time of renal biopsy. To be defined as significant, the

decline in GFR had to be greater than 10%. In addition, for a patient to be defined as a progressor, at the time of last follow-up their GFR had to be below the normal range (1.5-2.5 ml/sec). This was done to exclude patients who had a greater than 10% decline in GFR over time but still had a GFR within the accepted normal range at last follow-up. It was felt by the candidate that these patients should not be classed as progressors given that the decline in GFR may be explained by the natural decline in GFR associated with aging.

All patients who required renal replacement therapy were defined as progressors. This was necessary as a small proportion of patients presented with severe renal failure and their decline in GFR did not necessarily fit the inclusion criteria.

2.8.6 Statistical Analysis of Database

All statistical analysis of the database was performed using SAS V 8.0 (SAS Institute Inc, Cary, NC, USA). Creatinine concentration and 24-hour protein excretion were found to be well approximated using log-normal distributions and as such were log-transformed prior to analysis. Univariate analysis was conducted using t-tests and chi-square tests for equal proportion. Multivariate analysis was performed using multiple logistic regression with a stepwise selection technique and validated using a backward elimination process. A p-value of < 0.05 was considered to be statistically significant.

CHAPTER THREE

IGA NEPHROPATHY DATABASE

3.1 Introduction

In exploring the role of apoptosis in the progression of glomerulonephritis, particularly IgA nephropathy, it was critical to identify a large cohort of patients with biopsy proven disease. Not only did the patients have to be identified but it was also vital to demonstrate over a period of time, the course of disease in the identified patients. In view of what has already been discussed with regards to IgA nephropathy, although there *are* clinical and histological predictors of disease progression, each patient needs to be followed over several years, to reliably demonstrate the course of disease.

As disease parameters are well defined in glomerulonephritis, it is rare for an individual to undergo a renal biopsy without the collection of the appropriate clinical data. This allows for reliable retrospective collection of data for the majority of patients that have undergone renal biopsy even many years after the biopsy was performed. Furthermore, determining the course of disease in a given patient involves relatively simple blood and urine biochemical analysis. Thus most essential data required for the establishment of such a database is readily available or can be easily obtained.

In light of the significance of a diagnosis of IgA nephropathy, patients diagnosed with the disease are likely to remain in contact with their caring renal physicians for ongoing follow-up. This is true for most patients apart from those with very mild disease. Patients with very mild disease at presentation may not be as conscientious in maintaining follow-up as disease progression is unlikely and therefore this group are often under-represented in a database of a retrospective nature.

Given the nature of retrospective databases, it is often difficult to utilize them to examine new theories in disease progression. For example, the database set up by the candidate for IgA nephropathy extended back over a period of some 25 years. However the availability of the original renal biopsies for re-analysis as well as stored renal tissue, either in paraffin or frozen section, enables the application of new techniques to

biopsy tissue stored for many years. Furthermore, it is this group of patients in particular, those followed for many years, that will reveal most about the course of a disease that has been demonstrated to progress very slowly.

In summary, a database of patients with IgA nephropathy provides a powerful tool for demonstrating the course of disease in a patient population. The nature of the illness is such that it allows for reliable retrospective collection of data and the application of relatively straightforward testing to establish follow-up. The availability of stored biopsy tissue provides a unique opportunity to apply new research to a well described patient population.

3.2 Methodology

As per Section 2.8, all patients with primary IgA nephropathy diagnosed at The Alfred Hospital were identified. Patients with assumed secondary IgA nephropathy were excluded from analysis as were patients with HSP. Renal parameters for each patient at the time of biopsy were collected. Renal biopsy reports or where inadequate, renal biopsies themselves were examined to determine the degree of renal injury present. The scoring system described in Table 2.12 and 2.13 was used to quantify renal damage.

Those patients already being followed up as hospital outpatients or in private consulting rooms were identified and results of blood and urine biochemistry were obtained. If these had not been performed in the last 12 months arrangements were made with the patient or their caring physician for blood and urine samples to be collected.

Those patients lost to follow-up were contacted where possible and arrangements were made for physician review. At review, the collection of blood and urine samples was arranged.

The data collected was entered into a database and statistical analysis was performed to determine predictors of disease progression as described in Section 2.8.6.

It should be noted that data collected in this fashion allows for comparisons to be made between only two time points in the course of disease; at presentation and at last follow-up. Data collected in this manner does not allow for accurate determination of the rate of decline in renal function over time. Furthermore, it does not take into account

those parameters that tend to fluctuate over time such as blood pressure and the degree of proteinuria. As has been demonstrated by Bartosik et al [172] databases that collect information at several time points in the follow-up of IgA patients are much more reliable in determining those parameters associated with disease progression particularly when the *rate* of disease progression is measured. This method of analysis has highlighted the significance of blood pressure control over time in restricting the progression of IgA nephropathy.

Due to the retrospective nature of the database and the deficiency of available follow-up data for many patients, it was felt by the candidate that it would be more reliable if only a two time-point comparison was made.

3.3 IgA Nephropathy Database

A total of 175 patients with primary IgA nephropathy were identified. Of these, 158 patients were eligible for inclusion in the database as they had passed the minimum 2-year follow-up period. Of the 158 eligible patients, a further 21 patients could not be included in the database: 19 patients could not be located for follow-up, 1 patient had died without any follow-up and 1 patient declined to be involved in the study. A further 5 patients had died during the study period but were included in the analysis as they were known to have progressive disease at the time of death or had stable renal function at the 2-year minimum follow-up. Thus a total of 137 patients were able to be included in the database for analysis.

Baseline clinical parameters were available for all patients. Serum IgA was measured at the time of biopsy in only 55% of patients. Grading of renal injury was not possible for only three patients as biopsy reports as well as original biopsy sections were not available for analysis for these individuals. The average length of follow-up of the patients (minimum 2 years) was 60 months, with a range of 24-261 months.

Of the 137 patients with IgA nephropathy who had sufficient follow-up to be included for analysis, 48 (35%) patients had progressive disease and 88 (65%) had stable, non-progressive disease as defined in Section 2.8.5. Of the patients with progressive disease 25 patients or just over 50%, had reached end-stage renal failure requiring dialysis.

3.3.1 Age

The mean age of patients presenting with IgA nephropathy was 42 years (SEM ±1.18). It must be noted that The Alfred Hospital for the most part deals with adult medicine and thus it would be unlikely for a child with urinary abnormalities or renal failure to be referred to this institution. This obviously lends a significant bias to the mean age of patients included in the database. (Table 3.1)

The mean age of male patients was 41.7 years (SEM ±1.59) and female patients was 43 years (SEM ±2.21). This did not represent a significant difference. The mean age of patients with non-progressive disease was 39.8 years (SEM ±1.60) and with progressive disease was 48.2 years (SEM ±2.29). This did represent a significant difference ($p<0.005$), with those patients diagnosed with IgA nephropathy at an older age more likely to have progressive disease. (Table 3.3)

3.3.2 Patient Sex

The disease was almost three times more common in males than females with a ratio of 2.8:1. Only 16% of females had progressive disease compared with 42% of males. This difference was statistically significant ($p<0.01$). (Table 3.3)

3.3.3 Haematuria

Haematuria was defined as normal (<10 RBCx10^6/L), moderate (10-100 RBCx10^6/L) or severe (>100 RBCx10^6/L). Any patient with a history of macroscopic haematuria was classed as "severe" regardless of haematuria at the time of biopsy. Twenty-two percent of patients had no haematuria, 46% of patients had moderate haematuria and 32% of patients had severe or macroscopic haematuria. (Table 3.2)

Of note, patients with progressive disease had significantly lesser degrees of haematuria compared to patients with non-progressive disease ($p<0.05$). For example, only 20% of patients with progressive disease had heavy or macroscopic haematuria compared to 40% of patients with non-progressive disease.

3.3.4 Hypertension

Blood pressure at presentation was defined as "normal" if it was consistently below 130/90 mmHg, "moderate" if it ranged between 130-180/90-100 mmHg and

"severe" if it was consistently measured at greater than 180/100 mmHg or was associated with symptoms or signs consistent with malignant hypertension. The blood pressure classification for each patient was based on the range of blood pressures recorded around the time of the biopsy, regardless of any antihypertensive therapy.

Thirty-eight percent of patients were normotensive at the time of biopsy, 50% of patients had moderate hypertension and 10% of patients had severe or malignant hypertension (Table 3.2). There was a strong association between the degree of hypertension and disease progression ($p<0.0001$). Of note, 79% of patients with severe or malignant hypertension were shown to have progressive disease.

Baseline Clinical and Histological Characteristics	Mean (SEM)
Age	42 yrs (±1.18)
Male: Female	2.8:1
Serum creatinine	0.15 mmol/L (±0.01)
Creatinine clearance	1.52 ml/sec (±0.07)
Proteinuria	1.85 g/24-hours (±0.15)
Proliferative index	2.9 (±0.22)
Sclerosing index	4.9 (±0.19)
Overall renal injury score	7.8 (±0.26)

Table 3.1 Baseline characteristics for all patients with IgA nephropathy.

Baseline Clinical Characteristic	Normal (% of total)	Moderate (% of total)	Severe (% of total)	Association with Disease Progression
Haematuria	22	46	32	$p<0.05$
Hypertension	33	50	10	$p<0.0001$

Table 3.2 Baseline data for haematuria and hypertension at diagnosis.

3.3.5 Serum IgA

As mentioned, serum IgA concentration was measured at the time of biopsy in only 55% of patients. Of these patients, 55% had normal serum IgA (0.8-4.5g/L) at the time of biopsy and 45% had an elevated serum IgA (>4.5g/L).

A total of 28% of patients with non-progressive disease had elevated serum IgA levels compared to 31% of patients with progressive disease. This did not represent a statistically significant difference.

3.3.6 Renal Function at Presentation

Serum creatinine concentration was measured at the time of biopsy for all 137 patients. Creatinine clearance (CrCl) was measured by 24-hour urine collection at the time of biopsy for most patients. Where this was not available, CrCl was calculated using the Cockcroft-Gault formula [171]. The mean serum creatinine concentration for all patients at presentation was 0.15 mmol/L (SEM ±0.01). The mean CrCl for all patients was 1.52 ml/sec (SEM ±0.07). (Table 3.1)

The mean serum creatinine concentration for patients with non-progressive IgA nephropathy was 0.11 mmol/L (SEM ±0.007) and for patients with progressive disease was 0.28 mmol/L (SEM ±0.03). This difference was highly significant ($p<0.0001$). (Table 3.3)

Similarly CrCl was significantly greater in patients with non-progressive disease (mean 1.77 ml/sec SEM ±0.11) compared to that of patients with progressive disease (mean 0.87 ml/sec SEM ±0.09) ($p<0.0001$). (Table 3.3)

3.3.7 Proteinuria at Presentation

Proteinuria was measured for all patients at the time of biopsy. The mean protein excretion was 1.85 g/24-hours (SEM ±0.15) for all patients. (Table 3.1) Seven percent of patients had no proteinuria (<0.15 g/24-hours), 41% of patients had only mild proteinuria (0.15-1g/24-hours), 35% of patients had moderate proteinuria (1.0-3.5 g/24-hours) and 17% of patients had nephrotic range proteinuria (>3.5 g/24-hours). Overall 93% of patients had some degree of proteinuria (>0.15 g/24-hours) at presentation.

Patients with non-progressive disease had significantly less proteinuria (mean 1.39 g/24-hours SEM ±0.18) compared to patients with progressive disease (mean 3.22 g/24-hours SEM ±0.30) with a p value < 0.001. (Table 3.3)

Baseline Clinical Characteristics	Non-progressor Mean (±SEM) n=88	Progressor Mean (±SEM) n=48	P Value
Age (yrs)	39.8 (±1.59)	48.2 (±2.29)	<0.005
Male: Female	2.3:1	9:1	<0.01
Serum creatinine	0.11 mmol/L (±0.007)	0.28 mmol/L (±0.03)	<0.0001
Creatinine clearance	1.77 ml/sec (±0.11)	0.87 ml/sec (±0.09)	<0.0001
Proteinuria	1.39 g/24-hours (±0.18)	3.22 g/24-hours (±0.30)	<0.001

Table 3.3 Baseline clinical characteristics: progressors versus non-progressors.

3.3.8 Proliferative Index of Biopsy

As mentioned, changes seen on renal biopsy were divided into proliferative and sclerosing (see Section 2.8.4). This was an attempt by the candidate to divide the biopsy changes that are more usually associated with acute glomerular injury from those changes associated with chronic injury. Proliferative changes such as the formation of crescents or focal necrotizing lesions, although rare in IgA nephropathy, may represent part of an acute nephritic process that is potentially reversible. Conversely, those changes seen with chronic injury are usually thought to be irreversible. These include glomerulosclerosis as well as tubular atrophy and interstitial fibrosis.

The proliferative injury was scored out of a possible 12 points. (Table 2.12) For the most part proliferative injury observed in the renal biopsies was mild to moderate. The mean proliferative score for all patients was 2.9 (SEM ±0.22). (Table 3.1) Of note, severe proliferative injury was observed in 3 patients who presented with crescentic glomerulonephritis in association with acute renal failure.

The mean proliferative injury score for patients with non-progressive IgA nephropathy was 2.6 (SEM ±0.18). Patients with progressive IgA had a mean score of 3.46 (SEM ±0.30). This represented a significant difference ($p<0.02$). (Table 3.4)

3.3.9 Sclerosing Index of Biopsy

Sclerosing injury was similarly scored out of a possible 12 points. The degree of injury observed was quite variable with a mean score for all patients of 4.9 (SEM ±0.19) but a range of 1 to 12. (Table 3.1)

The mean score observed for patients with non-progressive IgA nephropathy was 3.9 (SEM ±0.20) and for patients with progressive disease was 7.6 (SEM ±0.26). This represented a highly significant difference ($p<0001$). (Table 3.4)

3.3.10 Overall Renal Injury Score

The proliferative injury score was combined with the sclerosing injury score to give an overall renal injury score out of 24. The mean overall renal injury score for all patients was 7.8 (SEM ±0.26). (Table 3.1)

The mean overall renal injury score for patients with non-progressive disease was 6.5 (SEM ±0.31) and for patients with progressive disease was 10.9 (SEM ±0.39). This represented a highly significant difference ($p<0.0001$). This was to be expected given the significant differences noted already in both the proliferative and sclerosing indices. (Table 3.4)

Renal Biopsy Parameters	Non-Progressors Mean (±SEM) n=88	Progressors Mean (±SEM) n=48	P Value
Proliferative index	2.6 (±0.18)	3.46 (±0.30)	<0.02
Sclerosing index	3.9 (±0.20)	7.6 (±0.26)	<0.0001
Overall renal injury score	6.5 (±0.31)	10.9 (±0.39)	<0.0001

Table 3.4 Summary of renal biopsy parameters: progressors versus non-progressors.

3.3.11 Glomerular IgA Deposition

Renal biopsies were graded for the intensity of glomerular IgA deposition observed within the glomeruli by immunohistochemistry or immunofluorescence. All patients had at least mild staining with IgA given that a diagnosis of IgA nephropathy is

dependant upon the presence of at least some IgA within the glomerulus. IgA staining was graded as mild, moderate or heavy.

Twenty-one percent of patients had only mild glomerular staining with IgA, 52% of patients had moderate IgA staining and 27% of patients had heavy staining with IgA. There was no significant relationship observed between the intensity of IgA staining and disease progression.

3.3.12 Glomerular IgG Deposition

Renal biopsies were assessed for the presence of IgG within glomeruli. The *intensity* of IgG staining was not noted, only its presence or absence. Seventy percent of patients with IgA nephropathy did not have significant IgG deposition within glomeruli. There was no significant association between the presence or absence of IgG staining within biopsies and disease progression.

3.3.13 Assessment at Most Recent Follow-Up

At follow-up, patients also underwent a 24-hour urine collection to assess proteinuria as well as a mid-stream urine to assess haematuria. This obviously was not possible for patients who had progressed to end-stage renal failure. In spite of this, there was a significant association between the degree of proteinuria at follow-up and progression of disease ($p<0.0001$). The mean proteinuria at follow-up for patients with non-progressive disease was 1.60g/24hrs (SEM ±0.08) compared to 2.6g/24hrs (SEM ±013) for patients with progressive disease. There was no association between the degree of haematuria at follow-up and disease progression.

It is interesting to note that the majority of patients with non-progressive disease, at last follow-up, still had urinary abnormalities. Up to 58% of non-progressors had either low-grade haematuria or mild to moderate proteinuria at follow-up. This was often after many years of relatively benign disease.

3.4 Predictors of Disease Progression by Multivariate Analysis

Using multivariate analysis it was possible to select those variables from the baseline patient characteristics and biopsy parameters that were significantly and *independently* associated with disease progression. This is in contrast to what has

already been observed on univariate analysis of the database. On univariate analysis, many of the parameters examined were demonstrated to correlate significantly with disease progression. However some of these parameters are likely to be dependant upon other variables within the database and therefore multivariate analysis was performed. For example serum creatinine concentration and CrCl are likely to be dependant upon the degree of renal injury observed at biopsy.

The results of the multivariate analysis revealed that only three of the variables measured were independent predictors of disease progression. These included in order of decreasing significance; sclerosing injury on biopsy, proteinuria at the time of biopsy and male sex. (Table 3.4)

Clinical or Biopsy Variable	P Value
Sclerosing injury on biopsy	<0.0001
Proteinuria at time of biopsy	<0.0001
Male sex	<0.02

Table 3.4 Variables significantly associated with progression of disease.

Several variables whilst significantly associated with disease progression on univariate analysis, proved not to be significant on multivariate analysis. These included patient age at biopsy, degree of hypertension, degree of haematuria, renal function at biopsy, proliferative index and overall renal injury score.

The multivariate analysis was performed based upon the assumption that sclerosing injury on biopsy and renal function at the time of biopsy were independent markers of disease progression. However, it is more likely that these parameters are inextricably linked to the progression of disease. The relationship between renal function, glomerular and tubulointerstitial sclerosis and disease progression in IgA nephropathy has been well documented [48]. Similar observations have been made for most other forms of chronic renal disease [173].

In light of this, the database was re-analysed for predictors of disease progression based on the assumption that renal function at biopsy and renal sclerosing

injury were surrogate markers of progressive disease. Using this method, the factors linked to disease progression were proteinuria at the time of biopsy, degree of hypertension at the time of biopsy, patient age and male sex. In other words, older male patients with heavy proteinuria at presentation were at the highest risk for renal injury and disease progression. (Table 3.5)

Clinical or Biopsy Variable	P Value
Proteinuria at time of biopsy	<0.0001
Age at biopsy	<0.003
Male sex	<0.02
Hypertension	<0.02

Table 3.5 Variables significantly associated with progression of disease, second analysis.

3.5 Discussion

It is reassuring to note that the conclusions drawn by the candidate from the analysis of the database match closely with observations made by most authors on disease progression in IgA nephropathy. The strong association between disease progression and proteinuria as well as renal sclerosing injury seen in this patient population on multivariate analysis concurs with D'Amico's conclusion in his meta-analysis of the available literature [5]. The weaker association between disease progression and male sex has also been observed by many other authors.

The secondary analysis of the database that was performed, based on the assumption that renal function at the time of biopsy and renal sclerosing injury were surrogate markers of progressive disease also concurred with observations made by other authors. Hypertension and age at the time of diagnosis of disease were shown to be significantly associated with disease progression as well as proteinuria and male sex on this multivariate analysis.

It could be argued that this assumption is not valid and that renal function at the time of biopsy is not in fact a surrogate marker of disease progression. However from previous discussions on the concept of "a point of no return" in IgA nephropathy, certainly in moderate degrees of renal impairment the two are inextricably linked. Few would argue that renal sclerosing injury on biopsy is not a surrogate marker of disease progression in most forms of glomerulopathy including IgA nephropathy. However, most of the published literature separates these two entities as independent variables, a practice that may be difficult to justify given previous observations.

As patient recruitment for the database extended over a period of 25 years there were a number of confounders that changed over this time period that may influence the makeup of the database as well as the overall rate of disease progression. The Renal Unit at The Alfred Hospital is made up of a group of physicians all with their own individual attitudes and thresholds for renal biopsies. As well as this, over the 25 years there has been some inevitable turnover in the attending renal physicians. This is certain to have had a significant but immeasurable impact on the make–up of the patient population.

Furthermore over this long period of time, newer therapies became available for treatment of hypertension, in particular ACE inhibitors, which have consistently been shown to alter the natural history of the disease [71]. Other specific therapies shown to alter the natural history of disease such as the use of corticosteroids [74], may have also influenced patient outcomes. Of note however, immunosuppressive therapy for IgA nephropathy has been rarely instituted at The Alfred Hospital as a matter of unit policy. These attitudes may well change if more evidence for the benefits of such therapies become available.

The overall rate of disease progression from this patient population was 35%, with half of these patients reaching end-stage renal failure. This is somewhat above the reported rates of disease progression of the order of 20% [5]. There are several possible explanations for this over-representation within the database of patients with more severe disease.

Firstly, as a major teaching hospital The Alfred Hospital is likely to attract patients with more severe renal disease and more associated complex medical problems. Secondly, of those patients lost to follow-up and excluded from final analysis, the majority had relatively benign disease and were not anticipated to progress. Finally, given that patients had to be followed for a minimum of two years with stable renal function to be classified as non-progressor, many patients biopsied in the last 3 years had to be excluded from the database despite anticipation that they would had relatively benign disease. Patients with clear progressive disease could be included in the database regardless of follow-up time, provide they satisfied the nominated criteria.

The biopsy scoring grades utilized were developed by the candidate. Most biopsy scoring systems in the literature follow similar lines but few attempt to differentiate between sclerosing injury and proliferative injury. Biopsy scoring systems, such as that used by Emancipator in his chapter on IgA nephropathy [28], tend to combine the two entities. Whilst distinct separation between sclerosis and proliferation may be at times artificial, the candidate was of the opinion that in a discussion of apoptosis in the pathogenesis of disease it may be useful to try and separate the two.

It has been informative to observe that proliferative changes on biopsy were not associated with progression of disease on multivariate analysis. This is distinct from sclerosing injury that was very strongly linked to disease progression. This is

noteworthy from a clinical as well as from a research perspective. Given that most of the patient received no specific therapy for their disease it may be that proliferative changes seen on biopsies are reversible even without treatment. Mesangial proliferation, crescents and focal necrotising lesions may be a transient phenomenon linked to the acute nephritic syndrome associated with a "flare of disease" accompanying an episode of macroscopic haematuria. Resolution of this acute inflammatory response is likely to be strongly linked to the process of apoptosis as has already been discussed.

The majority of patients in the database with primary IgA nephropathy had proteinuria at diagnosis, with 17% having nephrotic range proteinuria (>3g/24-hours). The reported incidence of nephrotic range proteinuria in IgA nephropathy is quite variable. In the 14 papers reviewed by D'Amico [5] the rate of nephrotic range proteinuria ranged form 1% to 33%. This variable rate of nephrotic range proteinuria is likely to be either a reflection of local biopsy thresholds or possibly a true difference in the incidence of heavy proteinuria between patient populations based on ethnicity and country of origin. Of note, Nicholls et al [174] reported on a large Australian cohort of patients with a rate of nephrotic range proteinuria of 5.3%. The rate in the candidate's patient population is somewhat higher than this at 17%, for what is likely to be a similar patient population. This suggests that the biopsy threshold in the candidate's population favours patients with heavy proteinuria. As discussed, this confounding factor was difficult to correct for given the retrospective nature of the data collection.

Finally in view of the above statements, one must consider the relationship between the onset of disease and the timing of the renal biopsy. As has been described in the reviewed literature, the biopsy changes seen with IgA nephropathy will vary depending on the relationship of the timing of the biopsy to the presenting symptoms. A biopsy close in time to an episode of macroscopic haematuria is more likely to reveal acute proliferative changes than one performed many months after the onset of symptoms. As this information was not readily available given the retrospective nature of the data collection, this aspect of the disease process could not be further analysed.

CHAPTER FOUR

IDENTIFICATION OF APOPTOTIC CELLS IN IGA NEPHROPATHY

4.1 Introduction

Initial studies which identified apoptotic cells in renal tissue were based on histological morphology alone. The first description of apoptotic cells in human renal tissue is accredited to Harrison [175] as recently as 1988, based only on light and electron microscopy findings. He observed apoptotic cells in biopsies from patients with diffuse proliferative glomerulonephritis. These cells were noted to contain small, hyperchromic fragments in association with condensation and fragmentation of the nucleus. Since these observations were made, a variety of methods have been developed to allow easier and more consistent recognition of cells undergoing apoptosis.

In 1992 Gavrieli et al [176] developed the technique described by the acronym TUNEL for better identification of apoptotic cells. It relies on the fact that the terminal event in apoptosis is nuclear DNA cleavage. This results in small fragments of DNA of variable length. The technique uses the enzyme - terminal deoxynucleotidyl transferase to attach dUTP-biotin to the free 3′ end of cleaved DNA. Then, using either immunofluorescence or immunohistochemistry, the attached dUTP-biotin is labelled and identified. In this way cells containing intact DNA fragments are highlighted, allowing identification of apoptotic cells in-situ. Since its development, this technique has been used extensively in human and animal tissues to study the occurrence and distribution of apoptotic cells in developmental, normal and diseased states.

4.2 TUNEL Methodology

The attraction of the TUNEL technique to the candidate was that it was readily applicable to formalin-fixed, paraffin embedded tissues. Given the retrospective nature of the study, only a small proportion of renal biopsies had segments frozen at the time of biopsy for future analysis. In fact, this only became the practice in the candidate's

laboratory in the mid 1990s with the development of PCR techniques. Thus a methodology had to be applied that could utilise stored, formalin-fixed, paraffin-embedded renal biopsy specimens. The TUNEL technique had been refined in the candidates laboratory for usage in such specimens and most patients who had undergone renal biopsy at The Alfred Hospital in the last thirty years had available a small amount of such stored tissue.

Unfortunately storage of renal biopsy samples over such a prolonged period of time also had its disadvantages. It quickly became apparent that a uniform pre-treatment for all tissue sections prior to performing the TUNEL technique was not going to be possible. Most likely as a result of the non-uniformity of specimen collection and fixation over such a long period of time, sections had to be individually tested with a variety of pre-treatments for optimum results. This certainly has implications for the reproducibility of the results obtained, as will be discussed.

Each microscope slide was prepared with two sections cut from a given renal biopsy. Where the tissue sample was large enough, one of the sections was divided into two, allowing for a negative and positive control. If the specimen was too small to be divided into two, a positive control only was performed as it was felt by the candidate that this was the more discriminating of the two possible controls. Given the scarcity of human biopsy tissue, it was felt that controls should be kept to a minimum, especially since the test specimen often had to be repeated to obtain a reliable result.

4.2.1 TUNEL Negative Control

As mentioned, where sufficient specimen was available a negative control was prepared for tissue undergoing the TUNEL technique. This involved incubation of the specimen without the enzyme - terminal deoxynucleotidyl transferase. In the absence of the enzyme, no cells were expected to be positive (nuclei staining dark brown) and the Haematoxylin stained nuclei dark blue. (Figure 4.1)

4.2.2 TUNEL Positive Control

Sections used as positive controls were pre-treated with DNase prior to performing the TUNEL procedure. This induced artificial fragmentation of nuclear DNA allowing the DIG-dUTP, in the presence of the enzyme, to bind to the free 3′ end

of cleaved DNA. For the positive control to be deemed adequate, at least 80% of the nuclei had to be stained brown. (Figure 4.1) If this was not the case, the process was repeated with a change in pre-treatment conditions until the desired result was achieved.

As a strong positive control confirmed that the TUNEL technique had been successful this had to be achieved before the test specimen could be interpreted. Once again the shortage of renal biopsy tissue did at times limit the number of pre-treatments that could be tested in order to achieve the optimum result.

4.2.3 TUNEL Test

The larger section of the two on each microscope slide was reserved for the test. Most sections were pre-treated with a short incubation in protease VIII at a concentration of 0.15mg/ml. Without pre-treatment, the positive control proved too weak and did not meet the criteria of 80% positivity as mentioned. The pre-treatment of specimens was altered to ensure a strong positive control that did not have excessive background staining. If background staining proved excessive, the concentration of protease VIII was reduced or the time spent incubating in the protease solution was reduced.

Although most specimens proved adequate when pre-treated at the initial concentration of protease, several specimens had to have pre-treatment adjusted on an individual basis. This had significant implications for the reproducibility of the results, although the usage of a positive control for each test sample ensured that individual test results were at least titrated to the same control.

Care had to be applied when identifying TUNEL positive cells within the test sample. As shown by Kockx et al [177] the TUNEL technique is not specific for cells undergoing apoptosis. Rather, it also identifies viable cells still undergoing active gene transcription. If one relies upon positive TUNEL staining *alone* to identify apoptotic cells, one would significantly overestimate the number of apoptotic cells in a given specimen. This fact is unfortunately not considered in many publications that use the TUNEL technique to identify apoptotic cells. For example, Kockx et al [177] demonstrated that a reliance upon TUNEL positivity alone may result in up to a thirty-fold overestimation of apoptotic cells. It is therefore considered necessary to combine TUNEL positivity with a further co-localizing system to identify true apoptotic cells.

In the candidate's case, cell morphology was combined with TUNEL positivity to more accurately estimate apoptotic cell number. Morphological changes treated as significant included cells with a condensed nucleus surrounded by clear cytoplasm and then a darkly staining rim, generating the so- called "halo effect". (Figure 4.1)

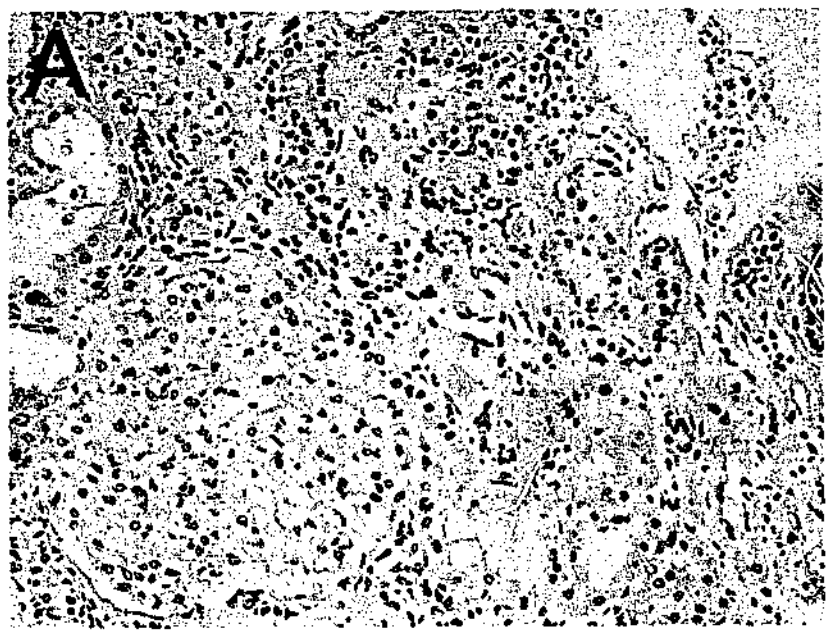
Apoptotic cells were identified and counted as described in Section 2.3.5. Tubulointerstitial apoptotic cells were expressed as TUNEL positive cells per mm^2. Glomerular apoptotic cells were expressed as number of TUNEL positive cells per glomerulus. Unfortunately whilst all biopsies contained a reasonable sample of the tubulointerstitium, several biopsies contained relatively few glomeruli. Of the 25 patient biopsy samples, two biopsies contained only 2 glomeruli, three contained only 1 glomerulus and four contained no glomeruli at all.

In view of the relative subjectivity involved in interpretation of the TUNEL stain, the candidate was blinded to the patient details at the time of counting.

Figure 4.1 A. TUNEL negative control sample. Note the light blue staining of nuclei.

B. TUNEL positive control sample. Note the dark brown staining of most nuclei.

C. TUNEL test sample. Note the TUNEL positive apoptotic cell in the centre (arrow) of the picture. Nucleus is condensed and dark brown, surrounded by clear cytoplasm and darkly staining rim generating the "halo effect".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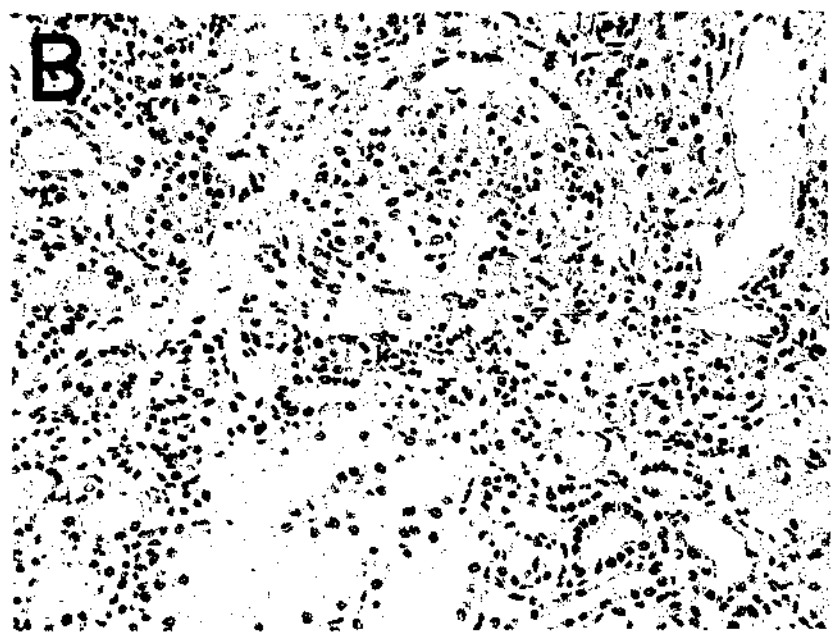
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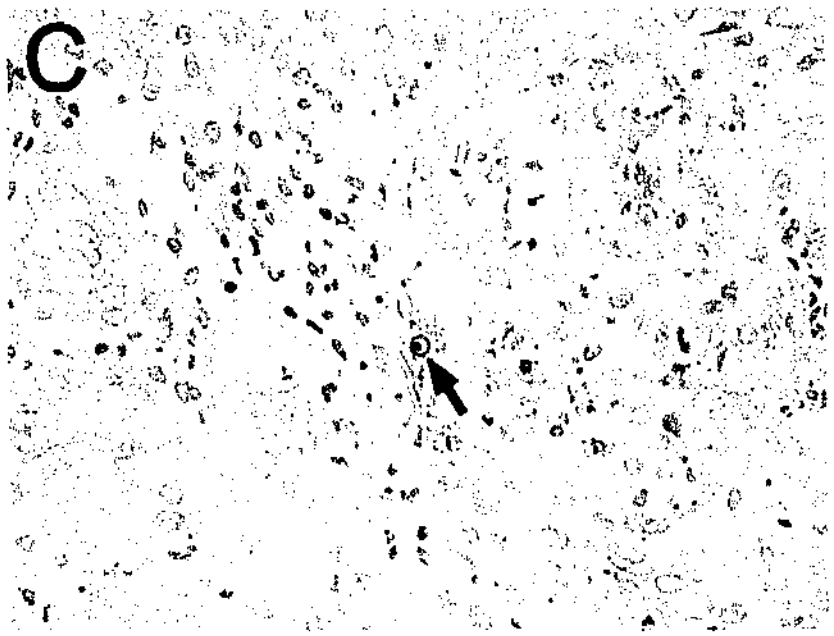
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4.3 Patient Details

A total of 30 biopsies were examined for the presence of apoptotic cells using the TUNEL technique. Five of the biopsies were from "normal" renal tissue for use as negative controls. "Normal tissue" was either from renal biopsies that had been examined by a senior histopathologist at The Alfred Hospital and deemed to represent normal renal tissue or from nephrectomy samples using normal tissue from the opposite pole of a renal tumour.

Twelve biopsies were from patients with non-progressive IgA nephropathy as defined in Section 2.8.5 (stable renal function at a minimum of two years follow-up). Thirteen biopsies were from patients known to have progressive IgA nephropathy as defined in Section 2.8.5 (decline in GFR of greater than 10% over time).

The mean age of patients with non-progressive IgA nephropathy was 35 years (SEM ±4.8) with a male to female ratio of 1.4:1. Mean serum creatinine concentration for this group was 86μmol/L (SEM ±4.0). Patients with progressive IgA nephropathy were on average older with a mean age of 52 years (SEM ±4.0) and a male to female ratio of 1.6:1. Mean serum creatinine concentration for this group was 315μmol/L (SEM ±48). Mean proteinuria was 0.93g/24hrs (SEM ±0.2) for patients with non-progressive disease and was 3.14g/24hrs (SEM ±0.6) for patients with progressive IgA nephropathy. The significant differences between the two groups (as shown in Table 4.1) are not surprising given the conclusions drawn from the analysis of the database as a whole. The data suggest that the two groups are reasonably representative of the two patient populations overall. (Table 4.1)

Similar differences were seen between the two patient groups in the sclerosing injury score on biopsy, with the progressors having significantly higher scores than non-progressors. Mean sclerosing injury score for non-progressors was 3.5 (SEM ±0.5) and for progressors was 8.2 (SEM ±0.4). The proliferative injury scores were similar for the two groups. (Table 4.1)

Clinical and Biopsy Characteristics	Non-Progressors Mean (±SEM) n=12	Progressors Mean (±SEM) n=13	P Value
Age	35 (±4.8)	52 (±4.0)	<0.01
Sex (M:F)	1.4:1	1.6:1	NS
Serum Creatinine (μmol/L)	86 (±4.0)	315 (±48)	<0.001
Proteinuria (g/24hrs)	0.93 (±0.2)	3.14 (±0.6)	<0.003
Sclerosing Injury Score	3.5 (±0.5)	8.2 (±0.4)	<0.0001
Proliferative Injury Score	2.5 (±0.34)	3.5 (±0.7)	NS

Table 4.1 Details of two patient groups for TUNEL analysis.

4.4 Statistical Analysis

Statistical analysis of the TUNEL results was performed using SAS V 8.0 (SAS Institute Inc, Cary, NC, USA). In view of the fact that positive cells per mm^2 and positive cells per glomerulus were found to be non-normally distributed, statistical significance between categorical variables was determined using a Kruskal–Wallis test. Significance between continuous, non-normally distributed data was ascertained using Spearman correlation coefficients. A p-value of < 0.05 was considered to be statistically significant.

Post-hoc comparisons between the three groups of patients studied were then performed using a Wilcoxon two-sample t-test. To account for multiple comparisons between the groups, a p value of < 0.02 was considered to be significant.

4.5 Results

Apoptotic cells were identified using the combination of TUNEL positivity and morphological features as described above. In normal tissue few apoptotic cells were identified in the tubulointerstitium with a mean of 0.02 cells/mm^2 (SEM ±0.01). Only a single glomerular apoptotic cell was identified per 150 glomeruli with a mean of

0.015/glomerulus. It is also important to note that the surface area of these specimens was far greater than that of the percutaneous biopsy specimens that were examined from patients with IgA nephropathy.

Whilst all specimens from patients with IgA nephropathy contained tubulointerstitial tissue, many sections had few if any glomeruli. Given that the all tissue studied were remnants of diagnostic renal biopsies this was not unexpected. Whilst statistical analysis of the results was still possible, it has been noted that 9 out of 25 patients had 2 or less glomeruli in total in their sections. Subsequent analysis was performed excluding those specimens that did not contain at least 3 glomeruli. This did not affect the significance of the results overall.

The mean number of tubulointerstitial apoptotic cells observed for all patients with IgA nephropathy, both progressors and non-progressors, was 0.13 cells/mm^2 (SEM ±0.04). This represented a significant increase in apoptotic cell number in patients with IgA nephropathy compared to normal controls ($p<0.02$). (Table 4.2 and Figure 4.2)

The mean number of glomerular apoptotic cells observed in all patients with IgA nephropathy was 0.25 cells/glomerulus (SEM ±0.14). This was not significantly higher than normal controls. (Table 4.2 and Figure 4.3)

With exclusion from analysis of those specimens that did not contain at least 3 glomeruli, the mean number of glomerular apoptotic cells observed in all patients with IgA nephropathy was 0.15 cells/glomerulus (SEM ±0.06). This was again not significantly different from normal controls.

Renal Compartment	Normal Controls n=5	IgA Nephropathy n=25	P Value
Tubulointerstitial Apoptotic cells/mm^2 Mean (±SEM)	0.02 (±0.01)	0.13 (±0.04)	$p<0.02$
Glomerular Apoptotic Cell no/glomerulus Mean (±SEM)	0.015	0.25 (±0.14)	NS

Table 4.2 Mean number of TUNEL positive cells; normal controls versus IgA nephropathy.

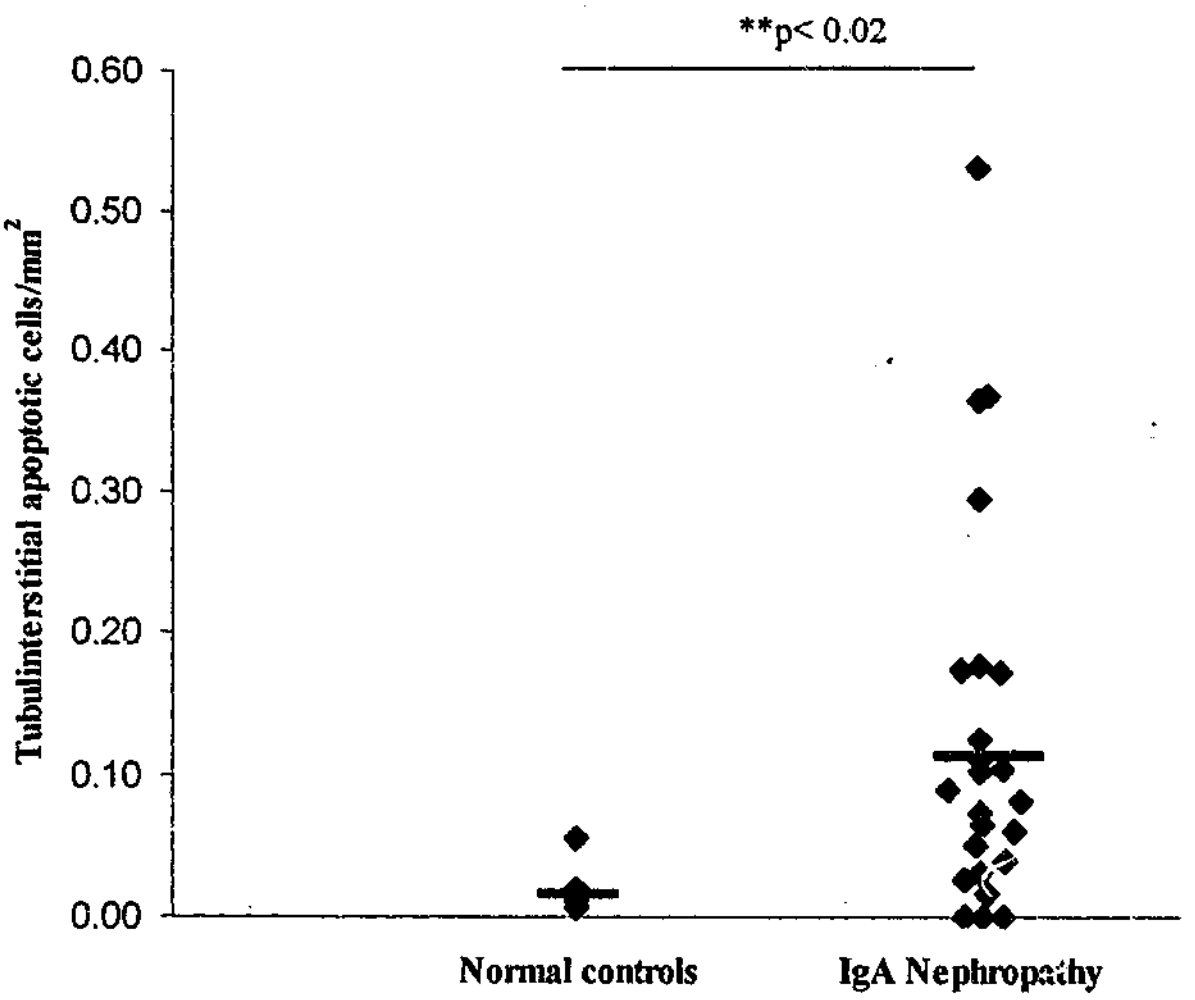


Figure 4.2 Tubulointerstitial apoptotic cells normal controls versus IgA nephropathy. Solid bars represent means. **A significant difference was observed ($p<0.02$).

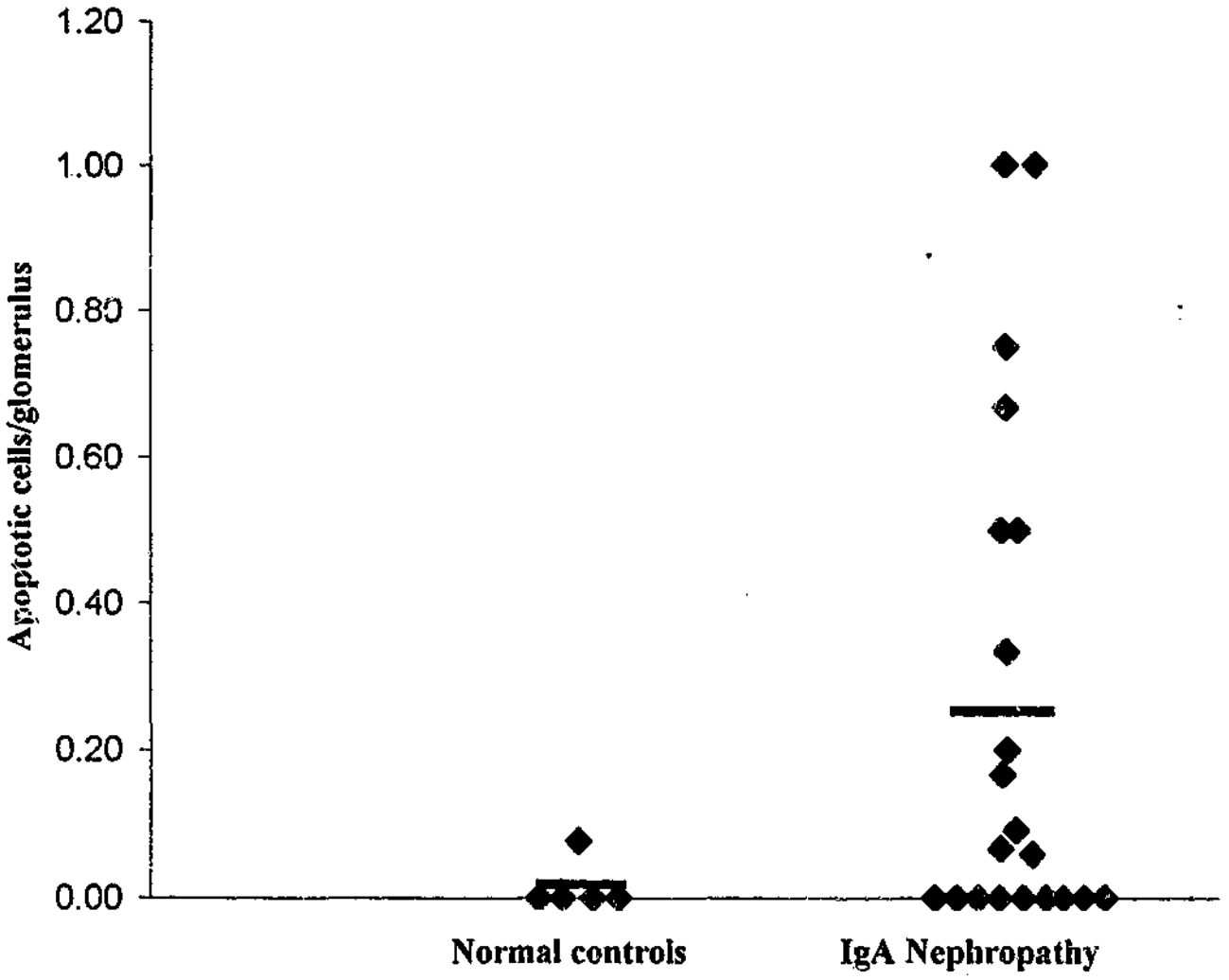


Figure 4.3 Apoptotic cells/glomerulus; normal controls versus IgA nephropathy. Solid bars represent means. No significant difference between the two groups was noted.

Comparison of apoptotic cell number was then made between patients with non-progressive IgA nephropathy and patients with progressive disease. In sections from patients with non-progressive IgA nephropathy the mean number of tubulointerstitial apoptotic cells was 0.05 cells/mm^2 (SEM ±0.01) compared to a mean number of 0.22 cells/mm^2 (SEM ±0.04) for patients with progressive IgA nephropathy. This represented a highly significant difference ($p<0.001$). (Table 4.3 and Figure 4.4)

Patients with non-progressive IgA nephropathy had a mean number of glomerular apoptotic cells of 0.09/glomerulus (SEM ±0.04) compared to a mean of 0.47/glomerulus (SEM ±0.14) for patients with progressive disease. (Table 4.3 and Figure 4.5) This did not represented a statistically significant difference ($p<0.05$) as for this section of analysis a p value of <0.02 was required to be considerer significant (see Section 4.4).

With exclusion from analysis of those specimens that did not contain at least 3 glomeruli, the mean number of glomerular apoptotic cells observed in patients with non-progressive disease was 0.06/glomerulus (SEM ±0.02) compared to a mean of 0.29/glomerulus (SEM ±0.14) for patients with progressive disease. This again did not represent a significant difference.

Renal Compartment	Non-progressive IgA Nephropathy n=12	Progressive IgA Nephropathy n=13	P Value
Tubulointerstitial Apoptotic cells/mm^2 Mean (±SEM)	0.05 (±0.01)	0.22 (±0.04)	<0.001
Glomerular Apoptotic Cell no/glomerulus Mean (±SEM)	0.09 (±0.04)	0.47 (±0.14)	NS

Table 4.3 Mean number of TUNEL positive cells; non-progressive IgA nephropathy versus progressive disease.

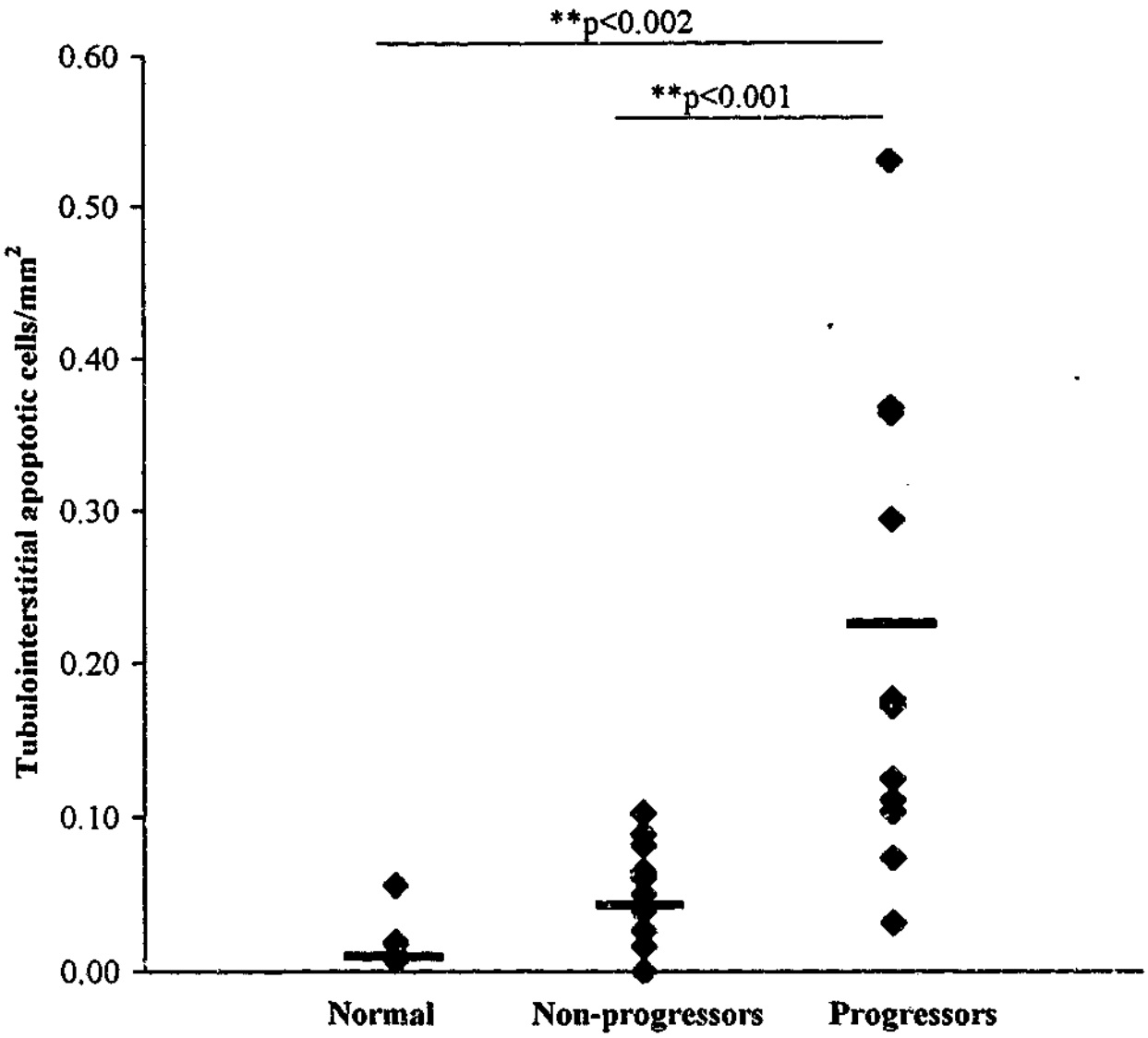


Figure 4.4 Tubulointerstitial apoptotic cell numbers per mm^2, normal versus non-progressors versus progressors. Solid bars represent means. **A significant difference between normal controls and progressors as well as between non-progressors and progressors was noted. No significant difference between normal controls and non-progressors was found.

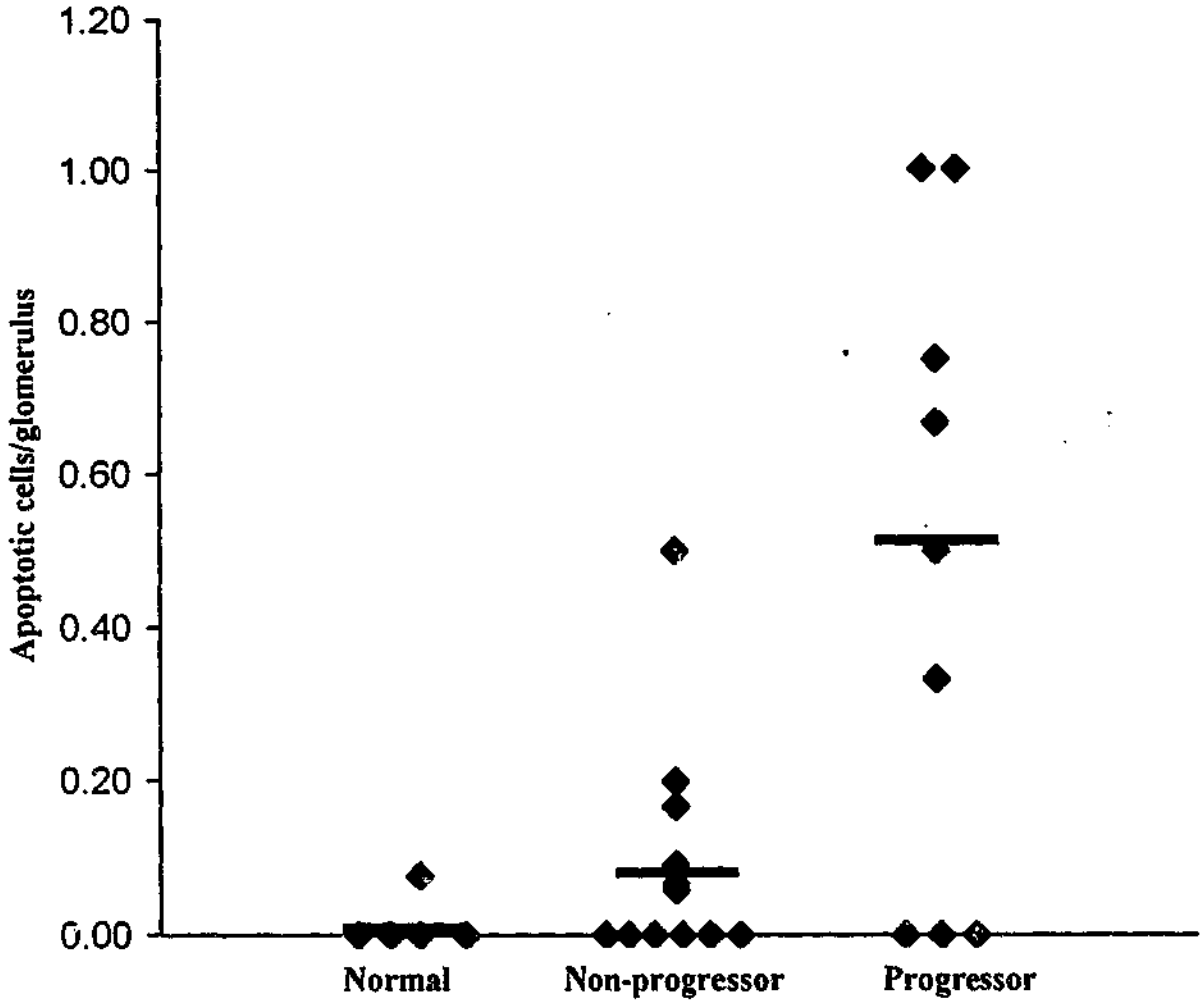


Figure 4.5 Apoptotic cell number per glomerulus, normal versus non-progressors versus progressors. Solid bars represent means. No significant differences were noted between the three groups.

Analysis was then made of the relationship between apoptotic cell number and the various known clinical and histological parameters for two patient groups. Tubulointerstitial apoptotic cell number was found to be significantly associated with serum creatinine ($p<0.0002$), sclerosing injury score ($p<0.002$) and proteinuria ($p<0.01$). This was not surprising given the association established from the database analysis between disease progression and these parameters. No association was demonstrated between tubulointerstitial apoptotic cell number and patient age, proliferative injury score and degree of haematuria. (Table 4.4)

Clinical and Biopsy Characteristics	**Tubulointerstitial Apoptotic Cell Number**
Age	NS
Sex (F>M)	NS
Serum Creatinine (μmol/L)	$p<0.0002$
Proteinuria (g/24hrs)	$p<0.01$
Sclerosing Injury Score	$p<0.002$
Proliferative Injury Score	NS
Degree of haematuria	NS

Table 4.4 Significant associations with tubulointerstitial apoptotic cell expression.

With respect to glomerular apoptotic cell number, a significant association was found with sclerosing injury score on biopsy ($p<0.02$). Of note there was no association between glomerular apoptotic cell number and proliferative injury score. No association with glomerular apoptotic cell number was found with patient age, sex, degree of proteinuria, serum creatinine and degree of haematuria. (Table 4.5)

Clinical and Biopsy Characteristics	Glomerular Apoptotic Cell Number
Age	NS
Sex (F:M)	NS
Serum Creatinine (μmol/L)	NS
Proteinuria (g/24hrs)	NS
Sclerosing Injury Score	$p<0.02$
Proliferative Injury Score	NS
Degree of haematuria	NS

Table 4.5 Significant associations with glomerular apoptotic cell expression.

There was no correlation between the number of glomerular apoptotic cells and the number of tubulointerstitial apoptotic cells.

4.6 Discussion

The observations made in this section of the candidate's thesis suggest a role for tubulointerstitial cell apoptosis in the progression of IgA nephropathy. The role of glomerular cell apoptosis in the progression of disease remains unclear from the observations made, but this may relate largely to the tissue samples examined and the relative deficiency of glomeruli available for analysis.

It is important to note that the sub-group of patients analysed were reasonably representative of the patient population being studied. The patients analysed for apoptotic cell numbers with non-progressive IgA nephropathy had minimal renal impairment and low grade proteinuria with sclerosing injury scores that were significantly lower than patients with progressive disease. Patients analysed with progressive IgA nephropathy had significant degrees of renal impairment with heavy proteinuria and significant sclerosing injury on biopsy. These difference between the

two groups examined were similar to those observed for the two groups in the analysis of the entire patient population (see Section 3.3).

The low number of apoptotic cells observed in normal human renal tissue by the candidate is similar to that observed by other authors [178]. This was noted in both the glomeruli and in the tubulointerstitial compartment. It is important to note that the sections of normal renal tissue examined were relatively large compared to the IgA nephropathy samples and thus the possibility of a sampling error for the normal tissue was low.

Glomerular apoptotic cell number was not significantly different between patients with IgA nephropathy and normal controls. Although the mean number of glomerular apoptotic cells was greater in patients with IgA nephropathy compared to controls, this difference was not statistically significant. Caution must be applied when interpreting these observations as many of the biopsy samples contained no or very few glomeruli. As the biopsy samples were for the most part only remnants of the original biopsies the amount of cortical tissue containing glomeruli was variable. It is thus difficult to draw firm conclusions from these observations. Certainly other authors have observed increased glomerular apoptotic cell number in patients with IgA nephropathy compared to controls in association with glomerular proliferation [128, 129].

On the other hand there was a clear increase in the number of tubulointerstitial apoptotic cells observed in patients with IgA nephropathy compared to controls. Few other published studies looking at human IgA nephropathy specifically examine the tubulointerstitium for apoptotic cells and therefore these observations are likely to be unique.

Comparisons between the two patient groups reveal a significant increase in the number of tubulointerstitial apoptotic cells in patients with progressive IgA nephropathy. Again, there is little in the published literature that specifically looks at these two patient groups focusing on the tubulointerstitial compartment. As the biopsies all contained reasonable samples of the tubulointerstitium these observations are likely to be significant.

It is possible that in patients with progressive IgA nephropathy there is activation of pathways that induce tubular cell and interstitial cell apoptosis giving rise to tubular atrophy and interstitial fibrosis. It is these changes that have been specifically

linked to disease progression in most forms of glomerulonephritis, including IgA nephropathy [31]. The possible factors that induce these tubulointerstitial changes in progressive IgA nephropathy will be further explored by the candidate in subsequent chapters.

In keeping with these observations, there was strong correlation between the number of tubulointerstitial apoptotic cells and renal function at the time of biopsy, proteinuria at the time of biopsy as well as sclerosing injury score. This is not surprising as these factors were all predictors of disease progression in IgA nephropathy. The link demonstrated between sclerosing injury on biopsy and tubulointerstitial apoptotic cell number suggest that these two processes are inextricably linked as has been demonstrated in animal models of glomerulosclerosis [132]. Again there is little in the published literature that examines human disease in this way.

Finally, the correlation between tubulointerstitial apoptotic cell number and the degree of proteinuria should be noted. This is quite significant as proteinuria is an independent marker of IgA nephropathy progression quite separate to the degree of sclerosing injury observed on biopsy. Whilst a relationship between tubulointerstitial atrophy and tubulointerstitial apoptosis would almost be anticipated, this significant correlation with proteinuria suggests this as a specific mechanism for tubular injury. As already discussed, some authors postulate that increased tubular protein traffic in glomerular disease is a potent inducer of tubular apoptosis and thus tubulointerstitial injury [155]. These observations made by the candidate seem to support this hypothesis.

Glomerular apoptotic cell number was increased in patients with progressive disease compared to non-progressors but this was not statistically significant ($p<0.05$). As mentioned, the paucity of glomeruli within the specimens examined makes it difficult to draw firm conclusions from this observation. Again, no other authors have specifically correlated glomerular apoptotic cell number with disease progression in IgA nephropathy or in other forms of glomerular disease for that matter, and thus this relationship still warrants further investigation.

CHAPTER FIVE

CELLULAR INFILTRATES AND THE EXPRESSION OF APOPTOTIC REGULATORS IN IGA NEPHROPATHY

5.1 Introduction

IgA nephropathy is characterised amongst other things, by glomerular hypercellularity. This increase in glomerular cell number is accompanied by an increase in mesangial cell matrix, the acellular material that supports the glomerular tuft. Whilst a wide variety of other histological changes are noted in IgA nephropathy, these two features are the two most consistently observed [28]. It is therefore highly likely that these two processes play an intrinsic role in the pathogenesis of disease. The factors that initiate mesangial proliferation and matrix expansion in IgA nephropathy remain poorly understood. Even more perplexing, is why in some patients these changes are only transient and resolve completely and in others there is a slow but progressive glomerulosclerosis and tubulointerstitial damage associated with a decline in renal function. It is clear that the processes that regulate acute glomerular inflammation and matrix production are likely to be crucial in understanding progressive IgA nephropathy.

Whilst specific glomerular changes are likely to *initiate* renal damage associated with glomerulonephritis, it is the non-specific changes seen within the tubulointerstitium that are more closely linked with disease progression. As early as 1968, Risdon et al [31] showed that in patients with chronic renal failure, renal function correlated most closely with tubular atrophy and interstitial fibrosis rather than glomerular changes. This suggests that it is the interplay between glomerular injury and tubulointerstitial injury, common to most forms of glomerulonephritis, which leads to irreversible disease progression. (One exception to this rule is minimal change glomerulonephritis in which despite acute glomerular injury, there is rarely any progression to tubulointerstitial injury if the condition is treated.)

In order to understand the acute inflammatory process in glomerulonephritis and in particular those factors that control its resolution or persistence, those cells involved in the inflammatory process must firstly be identified. Most experimental models of IgA nephropathy point to the mesangial cell as the major proliferating cell type. For example in Thy¹.1 nephritis it has been demonstrated that up to 85% of proliferating cells are mesangial in origin and the remaining cells are mostly monocyte-macrophages [179]. Human studies confirm these observations, but suggest a wider variety of non-mesangial cells including polymorphs and T-cells as well as monocyte-macrophages [180].

Similarly several studies have examined the nature of the inflammatory infiltrate associated with tubulointerstitial injury in both animal and human models of glomerulonephritis. For example Alexopoulos et al [30] identified monocyte/macrophages and T-cells as the predominant infiltrating cell type in the interstitium in 34 patients with IgA nephropathy. Most T-cells were $CD4^+$ and there was a significant correlation between renal function at the time of biopsy and the number of interstitial T-cells. Whether these cells are present as a *result* of tubulointerstitial injury or are the *cause* of cell injury and deletion remains to be established. Of note Shappell et al [181] have demonstrated that T-cell infiltration is not essential for the development of tubulointerstitial injury in a model using unilateral ureteric ligation in mice with severe combined immunodeficiency. This suggests that the tubulointerstitial cellular infiltrate seen in chronic, progressive glomerulonephritis may be a secondary phenomenon.

As mentioned, mesangial cell activation within the glomerulus is thought to give rise to the mesangial proliferation and increased mesangial matrix observed in IgA nephropathy. One important marker of mesangial activation is α-smooth muscle actin (α-SMA). Although the precise origin of mesangial cells remains unclear, it seems that the cells have similar origins to smooth muscle cells. With cellular activation there is an increase in the expression of α-SMA, one isoform of the intracellular microfilament actin. Normal human renal tissue expresses minimal α-SMA, but in a variety of proliferative glomerulonephropathies marked increased expression of α-SMA has been observed. Non-proliferative glomerulonephropathies do not demonstrate this increased expression, suggesting that α-SMA is an important indicator of mesangial cell activation

[182]. Kanko et al [183] have shown that glomerular α-SMA expression correlated significantly with disease activity in patients with IgA nephropathy.

This phenotypic change (expression of α-SMA) observed in mesangial cells in proliferative glomerulonephritis has also been observed in the tubulointerstitial myofibroblasts [182]. Experimental models of tubulointerstitial fibrosis demonstrate that tubular epithelial cells undergo phenotypic transformation associated with increased expression of α-SMA leading to local tubulointerstitial fibrosis [184].

The candidate has demonstrated in the previous chapter that apoptosis of glomerular and interstitial cells *does* occur in IgA nephropathy. This is increased in patients with progressive disease compared to patients with non-progressive disease particularly in the tubulointerstitial compartment. It remains to be demonstrated what factors are regulating apoptosis in IgA nephropathy and which cells in particular are involved in the process.

The purpose of the experiments described in this chapter was to firstly characterise the glomerular and interstitial infiltrates associated with IgA nephropathy and to compare the pattern of infiltrating cells between patients with progressive and non-progressive disease. Similarly glomerular and interstitial α-SMA expression was measured as a marker of mesangial cell proliferation [185] and interstitial myofibroblast infiltration [186].

Finally biopsy sections were examined for the expression of some of the proteins that are known to regulate apoptosis. These included Bax, Bcl-2, Bcl-X_L and Fas-ligand. The expression of these proteins was then correlated with IgA nephropathy progression as well as with patient clinical and histological parameters.

Immunohistochemistry, as outlined in Section 2.5, was used for identification of inflammatory cell types, the expression of α-SMA and the detection of the regulatory proteins of apoptosis.

5.2 Negative Controls

To control for any non-specific background staining, each batch of sections used for immunohistochemistry included a diluent negative sample. This section was incubated with diluent alone in the absence of any immunoglobulin. To control for any non-specific staining associated with the immunoglobulin type, each batch of sections

also included an isotype control. This included IgG1 and IgG2a to control for the two antibody isotypes used in the experimentation. There was no non-specific staining observed with either form of negative control.

Normal nephrectomy samples were not utilised in this section of the thesis as normal controls. This was due to a lack of sufficient normal renal tissue samples to perform this analysis reliably. However it is clear from the literature that normal renal tissue has few inflammatory cells present within glomeruli or the interstitium [28] and expresses minimal α-SMA [182]. As is demonstrated in the coming chapters, in normal tissue mRNA expression of Fas, Fas-ligand and Bcl-2 is also minimal.

5.3 Cellular Infiltrates

In order to identify the inflammatory cell-type associated with glomerular and interstitial infiltrates the following cell markers were applied to sections from patients with IgA nephropathy. Positive cells were counted and expressed per mm^2 for interstitial infiltrates and per glomerulus for glomerular infiltrates (see Section 2.5.6). Pre-treatment with citrate buffer was applied to some sections prior to the application of specific immunoglobulins as described in Section 2.5.4.2.

5.3.1 Leucocyte Common Antigen (LCA)/CD45

Monoclonal mouse anti-human LCA/CD45 (Zymed Laboratories) was used to identify all white blood cells present. LCA/CD45 is located on the surface of all human white blood cells including lymphocytes and polymorphs. (Figure 5.1)

5.3.2 CD3

Monoclonal mouse anti-human CD3 (Novocastra) was used to identify T-cells within glomeruli and interstitial infiltrate. The CD3 molecule is associated with the T-cell antigen receptor complex and is present on the surface of over 90% of T lymphocytes. (Figure 5.1)

5.3.3 L-26/CD20

Monoclonal mouse anti-human CD20 (Dako) was used to identify B-cells within glomeruli and interstitial infiltrate. CD20 is a polypeptide present on the surface of the

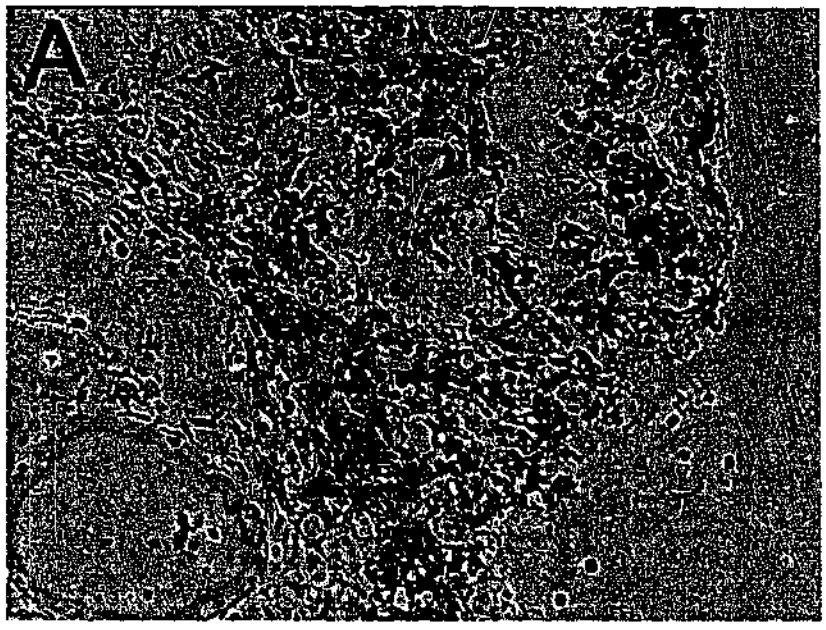
majority of B-cells. The L26 epitope was utilised, as it is resistant to formalin fixation. (Figure 5.2)

5.3.4 CD68

Monoclonal mouse anti-human CD68 antibody (Dako) was used to identify infiltrating macrophages within glomeruli and interstitial infiltrate. CD68 is a transmembrane protein of unknown function, located on the surface of human macrophages. (Figure 5.2)

Figure 5.1 A. An example of a leucocyte common antigen/CD4 positive infiltrate in a section from a patient with progressive IgA nephropathy.

B. An example of CD3 positive cells (T-cells) within the infiltrate in the same patient, serial section.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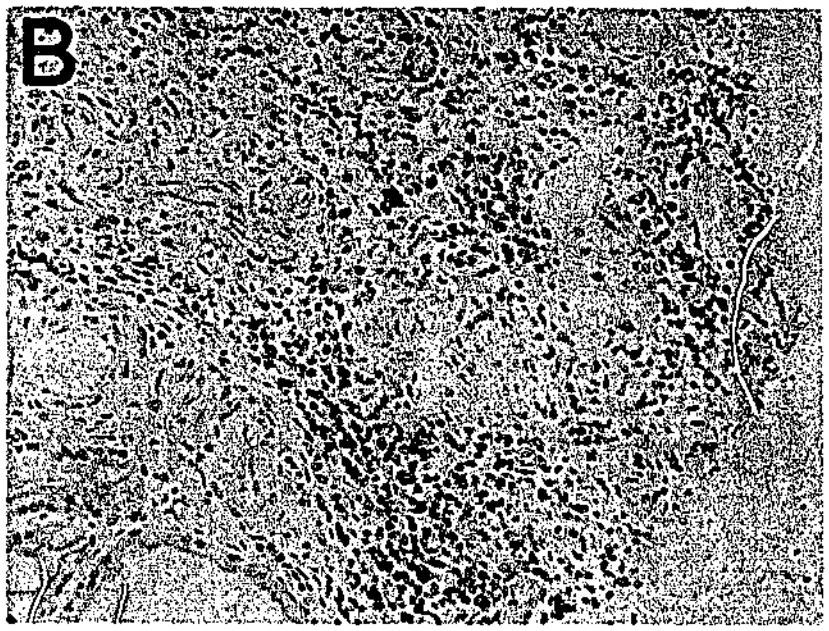
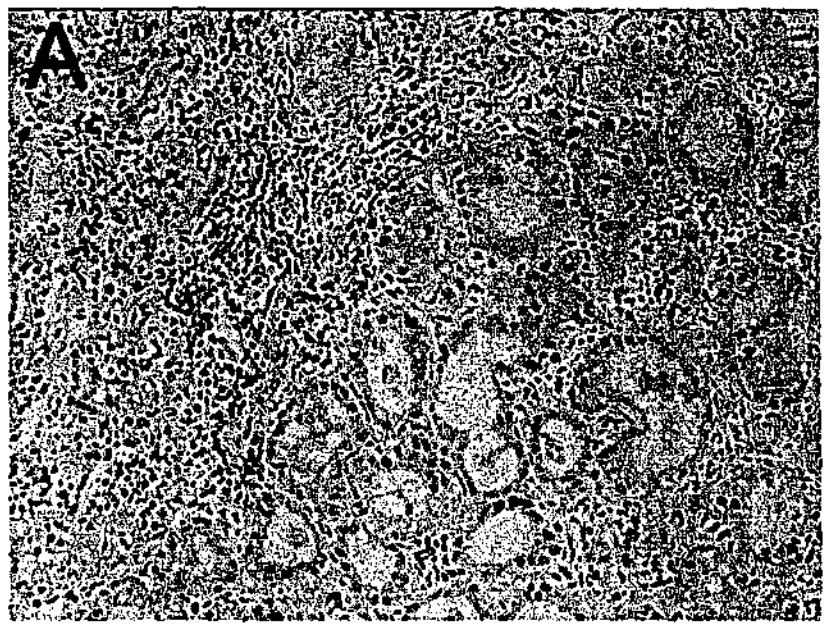

B

Figure 5.2 A. An example of CD20 positive cells (B-cells) in a patient with progressive IgA nephropathy.

B. An example of CD68 positive cells (macrophages) within the infiltrate in the same patient as Figure 5.1 A and B, serial section.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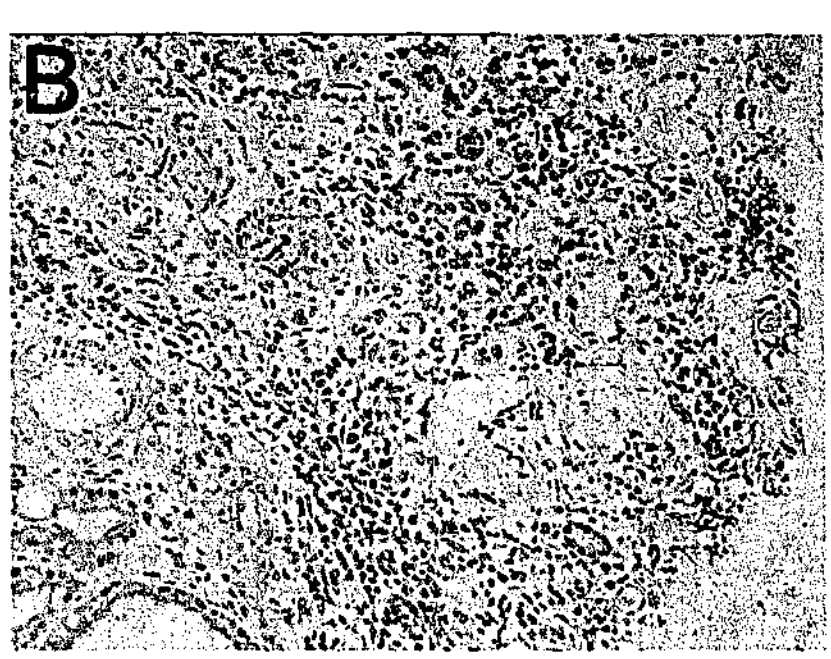
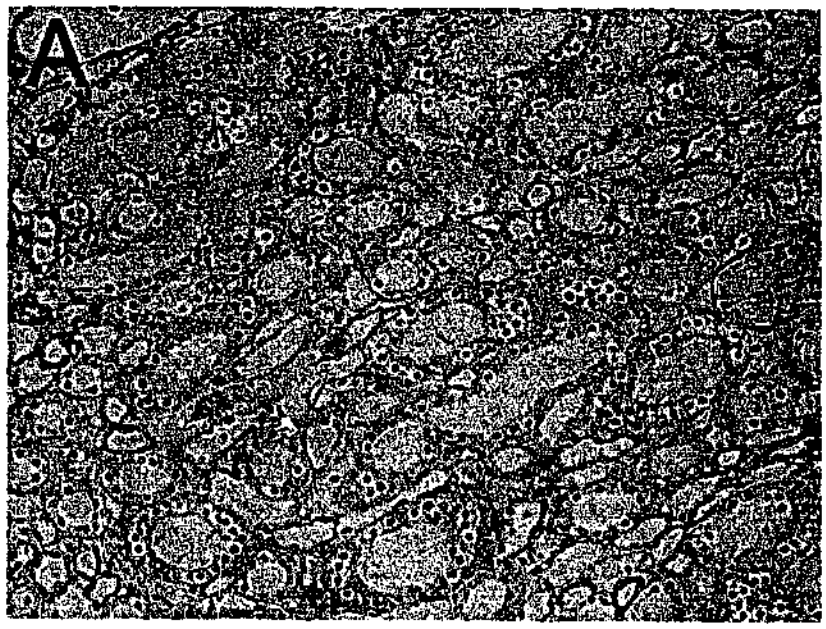

B

5.4 Alpha-Smooth Muscle Actin (α-SMA)

Mouse monoclonal anti-human α-SMA (Sigma) was used to identify activated glomerular mesangial cells as well as activated interstitial myofibroblasts. The antibody is specific for the single isoform α-actin. Expression of α-SMA within the glomerulus was graded from 0 to 3 with 0 representing no positive staining, 1 representing mild staining, 2 representing moderate staining and 3 representing heavy staining. Similarly, tubulointerstitial α-SMA expression and peri-glomerular α-SMA expression was graded from 0 to 3. (Figure 5.3)

Figure 5.3 A. Tubulointerstitial expression of α-SMA in a section from a patient with non-progressive IgA nephropathy.

B. Tubulointerstitial expression of α-SMA in a section from a patient with progressive IgA nephropathy.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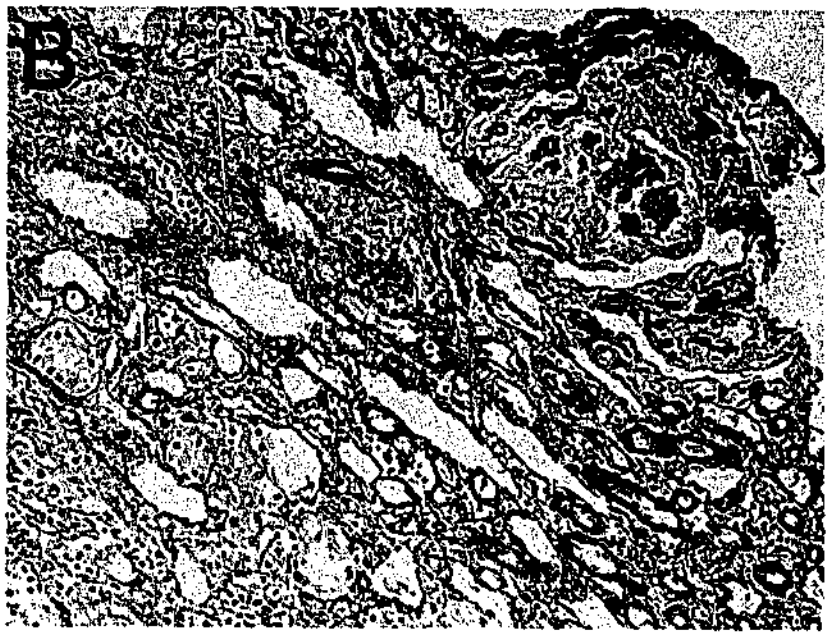

B

5.5 Regulators of Apoptosis

Along with identifying the components of cellular infiltrates in biopsies from patients with IgA nephropathy, the candidate attempted to measure the expression of some of the regulators of apoptosis. The intensity of expression as well as the distribution of these regulators was noted. To measure the cellular expression of Bax and Bcl-2, positive cells were counted and expressed per mm^2. The expression of Fas-ligand and Bcl-X_L was graded from 0 to 3 with 0 representing no positive staining, 1 representing mild staining, 2 representing moderate staining and 3 representing heavy staining. Pre-treatment with citrate buffer was applied to some sections prior to the application of specific immunoglobulins as described in Section 2.5.4.2.

5.5.1 Bax

Monoclonal mouse anti-human Bax (Zymed Laboratories) was used to identify the expression of this protein. The Bax protein forms a heterodimer with anti-apoptotic proteins, in particular Bcl-2 to induce apoptosis. The antibody used reacted with all known isoforms of human Bax. (Figure 5.4)

5.5.2 Bcl-2

Monoclonal mouse anti-human Bcl-2 (Dako) was used to measure the expression of this anti-apoptotic protein. The Bcl-2 protein lies within the cell and is associated with intra-cellular organelles such as the mitochondria and smooth endoplasmic reticulum. It plays a pivotal role in the inhibition of cell death. (Figure 5.4)

5.5.3 Bcl-X_L

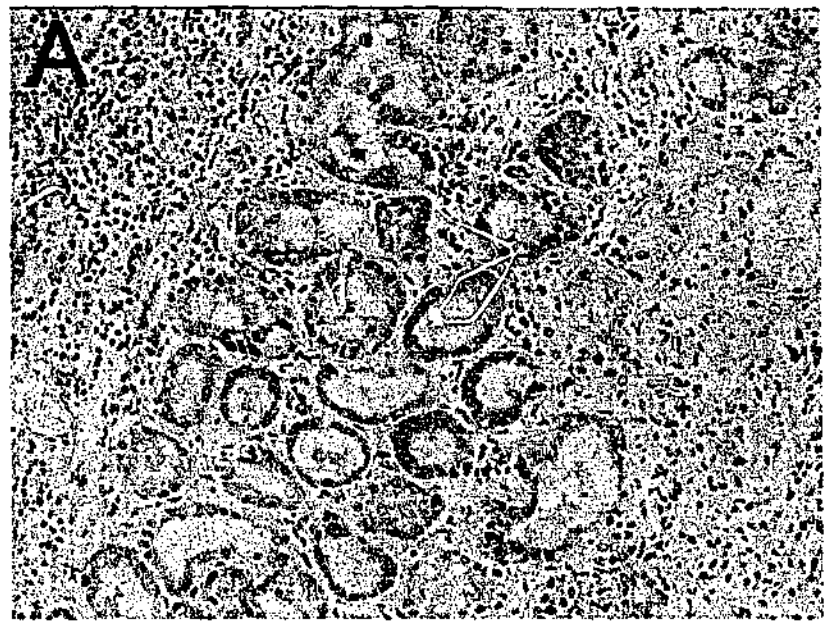
To measure the expression of Bcl-X_L, the candidate used monoclonal mouse anti-human Bcl-X_L (Zymed Laboratories). (Figure 5.5) Of the two isoforms of Bcl-X, Bcl-X_L is the longer (hence the subscript L) and shares significant homology with Bcl-2. It plays a similar role in the inhibition of apoptosis. The other isoform of Bcl-X, the shorter Bcl-X_S, is a pro-apoptotic protein.

5.5.4 Fas-Ligand

Fas-ligand is a member of the tumour necrosis factor (TNF) family of proteins. When Fas-ligand is cross-linked with the membrane protein Fas, also a member of the TNF family, it acts as a potent inducer of apoptosis. To study the expression of this protein, the candidate used monoclonal mouse anti-human Fas-ligand (Transduction Laboratories). (Figure 5.5)

Figure 5.4 A. An example of Bax staining in a patient with progressive IgA nephropathy. Expression of Bax was noted in association with infiltrating leucocytes as well on tubular epithelial cells.

B. An example of Bcl-2 staining in a patient with progressive IgA nephropathy. Expression of Bcl-2 was observed in association with infiltrating leucocytes with no tubular expression.


A

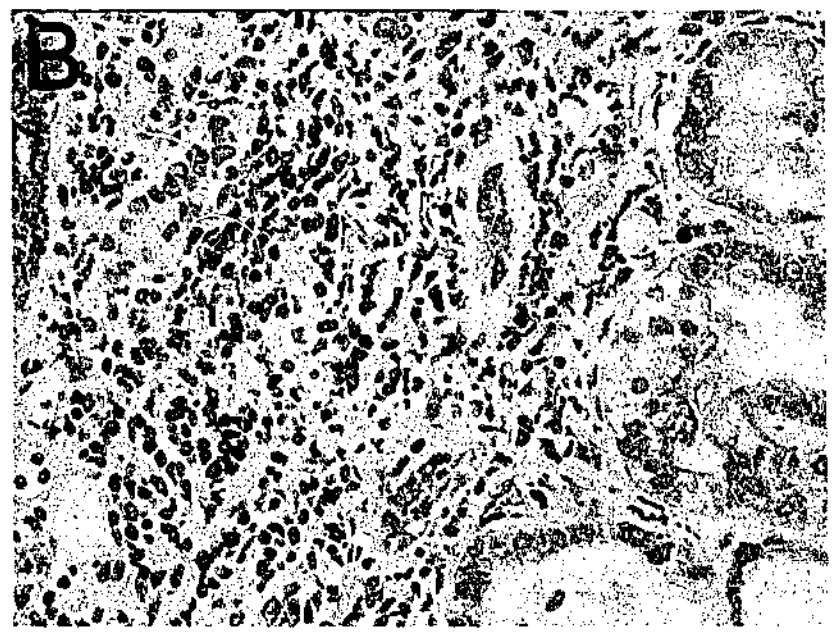
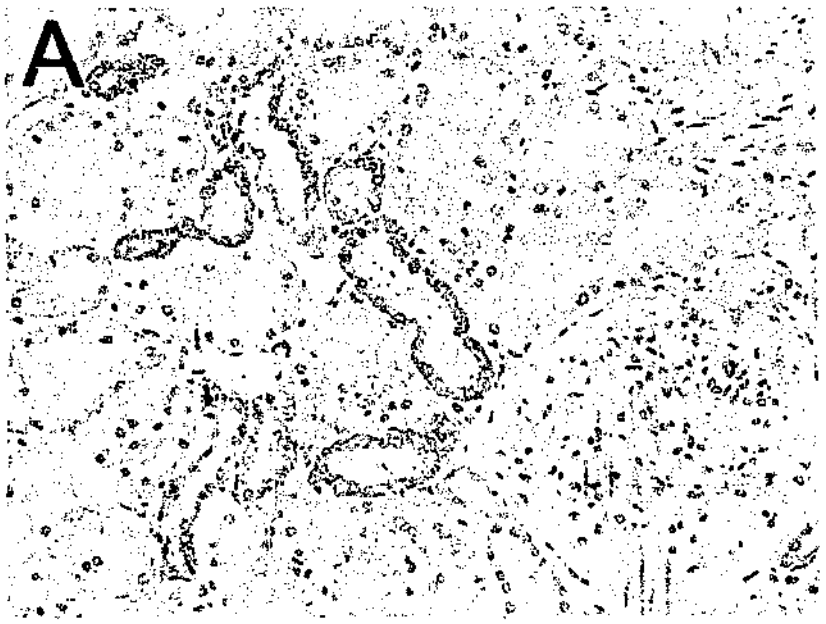

B

Figure 5.5 A. An example of Bcl-X_L staining in a patient with progressive IgA nephropathy. Bcl-X_L was expressed on tubular epithelial cells only.

B. An example of Fas-ligand staining in a patient with progressive IgA nephropathy. Fas-ligand was expressed on tubular epithelial cells only.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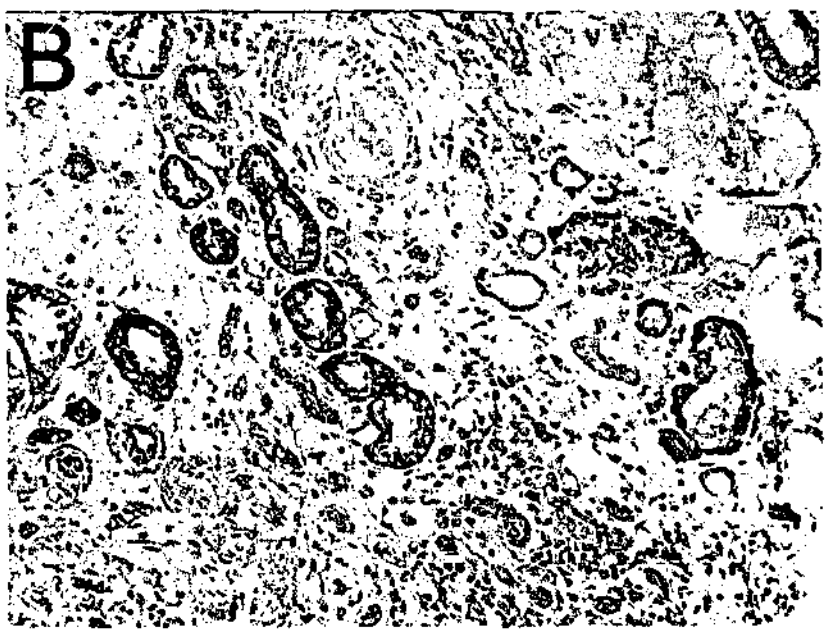
B

5.6 Patient Details

Biopsies from a total of 22 patients with IgA nephropathy were examined. Nine patients were known to have stable, non-progressive disease on follow-up (stable renal function at a minimum follow-up of two years) and 13 patients had progressive renal impairment (decline in GFR of greater than 10% over time) as defined in Section 2.8.5.

Three of the nine patients with non-progressive disease and four of the thirteen patients with progressive disease had been analysed in the previous chapter for the expression of apoptotic cells. Ideally all patients from the previous chapter would have been utilised for this section of experimentation however there was insufficient renal tissue for many of the patients for this to occur.

The mean age of patients with non-progressive IgA nephropathy was 40 years (SEM ±5.2) and for progressors was 45 years (SEM ±4.2). This did not represent a significant difference. The male to female ratio for non-progressors was 2:1 and for progressors was 12:1. Serum creatinine concentration was significantly lower in patients with non-progressive disease with a mean of 94μmol/L (SEM ±4.5) compared to progressors with a mean of 166μmol/L (SEM ±20) ($p<0.01$). (Table 5.1)

Mean proteinuria was 1.92g/24hrs (SEM ±0.84) for patients with non-progressive disease and was 3.66g/24hrs (SEM ±0.74) for patients with progressive IgA nephropathy. This difference was not statistically significant ($p<0.07$). Progressors had significantly higher sclerosing injury scores (mean 6.92 SEM±0.52) than non-progressors (mean 3.44 SEM±0.28) but proliferative injury scores were similar for the two groups. (Table 5.1) Again, the differences observed between progressors and non-progressors were similar to those observed between the two groups in the overall patient population.

Clinical and Biopsy Characteristics	Non-Progressors Mean (±SEM) n=9	Progressors Mean (±SEM) n=13	P Value
Age	40 (±5.2)	45 (±4.2)	NS
Sex (M:F)	2:1	12:1	<0.001
Serum Creatinine (μmol/L)	94 (±4.5)	166 (±20)	<0.01
Proteinuria (g/24hrs)	1.92 (±0.84)	3.66 (±0.74)	NS
Sclerosing Injury Score	3.44 (±0.28)	6.92 (±0.52)	<0.001
Proliferative Injury Score	3.66 (±0.72)	3.53 (±0.59)	NS

Table 5.1 Details of two patient groups for immunohistochemical analysis.

5.7 Statistical Analysis

Statistical analysis of the results of this section was performed using SAS V 8.0 (SAS Institute Inc, Cary, NC, USA). In view of the fact that most cell number counts and scores were found to be non-normally distributed, statistical significance between progressors and non-progressors was determined using a Wilcoxon two sample t-test for non-parametric variables. For similar reasons, Spearman correlation coefficients were utilized to assess any significant relationship between all variables examined. A p-value of < 0.05 was considered to be statistically significant.

5.8 Results

5.8.1 Glomerular Infiltrates

For patients with non-progressive IgA nephropathy, the mean glomerular leucocyte count was 5 cells per glomerulus (SEM ±1.05). The mean macrophage count was 1.25 cells per glomerulus (SEM ±0.43). Thus approximately 25% of infiltrating glomerular leucocytes were macrophage in origin.

For patients with progressive disease, a similar number of infiltrating glomerular leucocytes was observed, the mean being 4.14 cells per glomerulus (SEM ±0.72). The

mean number of glomerular macrophages was 0.88 cells per glomerulus (SEM ±0.21), indicating that in this group of patients approximately 21% of infiltrating glomerular leucocytes were macrophage in origin. These differences between the two groups in both leucocyte infiltration and macrophage number were not statistically significant (see Figure 5.6 and 5.7).

There were no T or B-cells identified within the glomeruli of either non-progressors or progressors, suggesting that the remaining infiltrating leucocytes within the glomeruli were predominantly polymorphs.

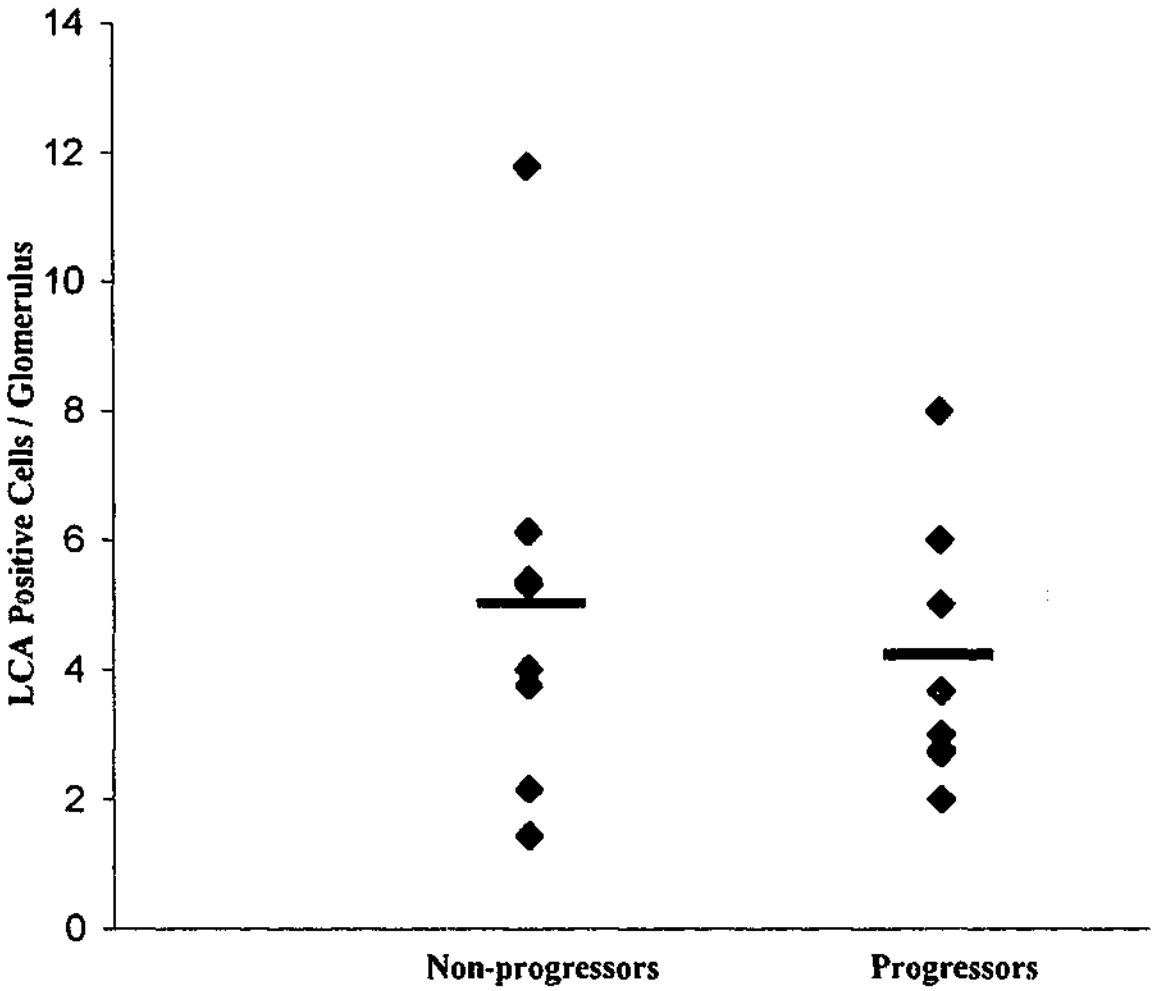

Figure 5.6 Expression of LCA/CD45 (leucocytes) in glomeruli, non-progressors versus progressors. Solid bars represent means. No significant difference was found.

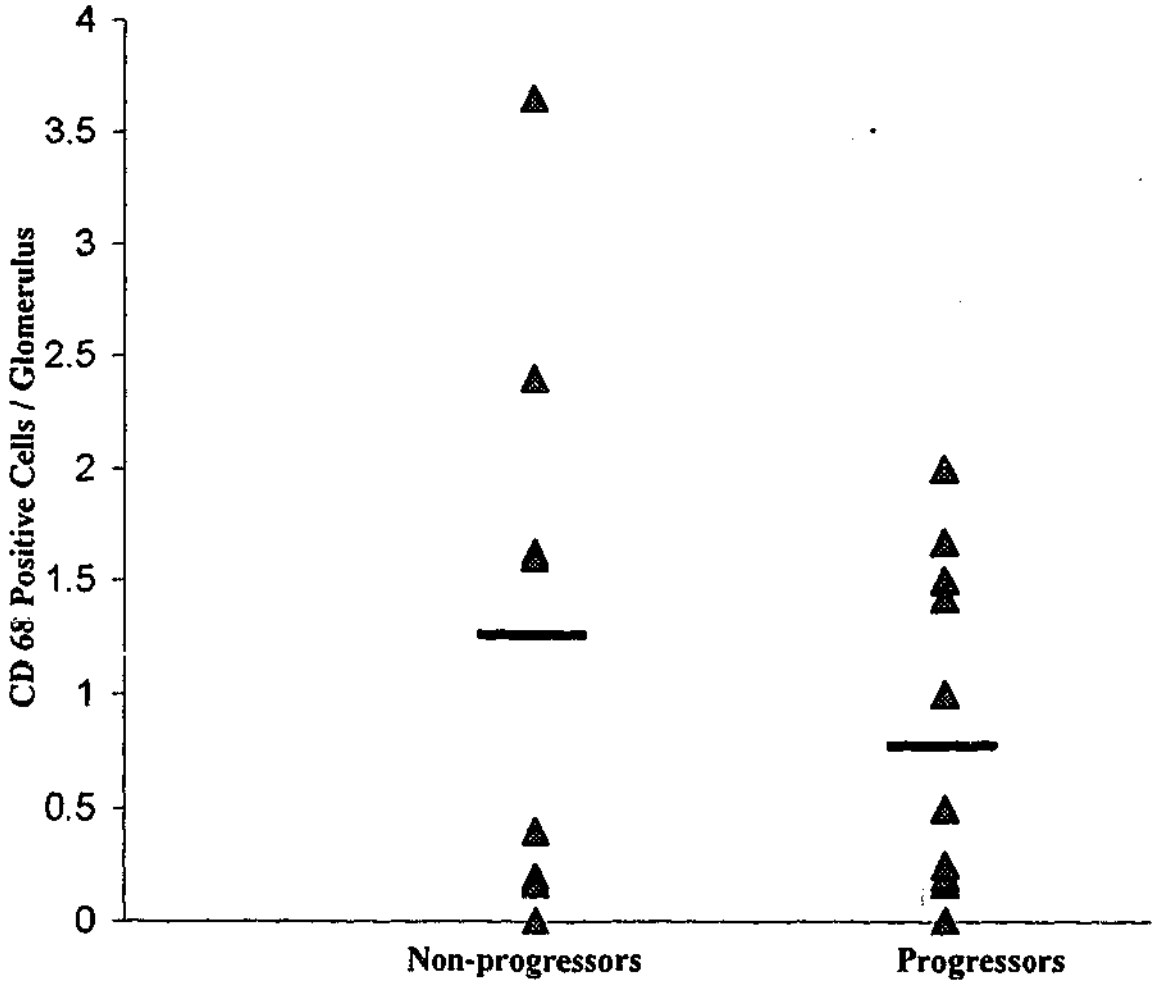

Figure 5.7 Expression of CD68 (macrophages) in glomeruli, non-progressors versus progressors. Solid bars represent means. No significant difference was found.

Of note the glomerular leucocytic infiltrate as revealed by LCA expression was not found to correlate significantly with the proliferative injury score on biopsy ($p=0.29$). Similarly there was no significant correlation between macrophage number and proliferative injury score. This supports what has already been discussed, that the predominant proliferating cell within the glomerulus in IgA nephropathy is the mesangial in origin [179].

5.8.2 Interstitial Infiltrates

The mean number of total leucocytes (LCA/CD45) in the interstitium of patients with non-progressive IgA nephropathy was 37.3 cells per mm^2 (SEM ±6.27). The mean number of B-cells was 2.9 cells per mm^2 (SEM ±0.64), T-cells was 16.9 cells per mm^2 (SEM ±3.96) and macrophage was 1.3 cells per mm^2 (SEM ±0.26). (Table 5.2) Eighty per-cent of infiltrating cells were T-cells, with B-cells comprising of 14% and macrophages 6 per cent.

For patients with progressive disease the mean number of infiltrating leucocytes was 60 cells per mm^2 (SEM ±12.09). The mean number of B-cells was 8.5 cells per mm^2 (SEM ±1.82), T-cells was 34.5 cells per mm^2 (SEM ±8.0) and macrophages was 1.8 cells per mm^2 (SEM ±0.42). (Table 5.2) Thus 77% of the infiltrating cells were T-cells, 19% were B-cells and 4% were macrophages.

Only the difference in B-cell numbers between non-progressors and progressors was found to be significant ($p<0.05$). (Figure 5.8)

Cell Type	Non-progressor cells/mm^2 Mean (±SEM) n=9	Progressor cells/mm^2 Mean (±SEM) n=13	P Value
Total leucocytes	37.3 (±6.27)	60 (±12.09)	NS
T-cell	16.9 (±3.96)	34.5 (±8.00)	NS
B-cell	2.9 (±0.64)	8.5(±1.82)	$p<0.05$
Macrophage	1.3 (±0.26)	1.8 (±0.42)	NS

Table 5.2 Interstitial infiltrating cells; progressors versus non-progressors.

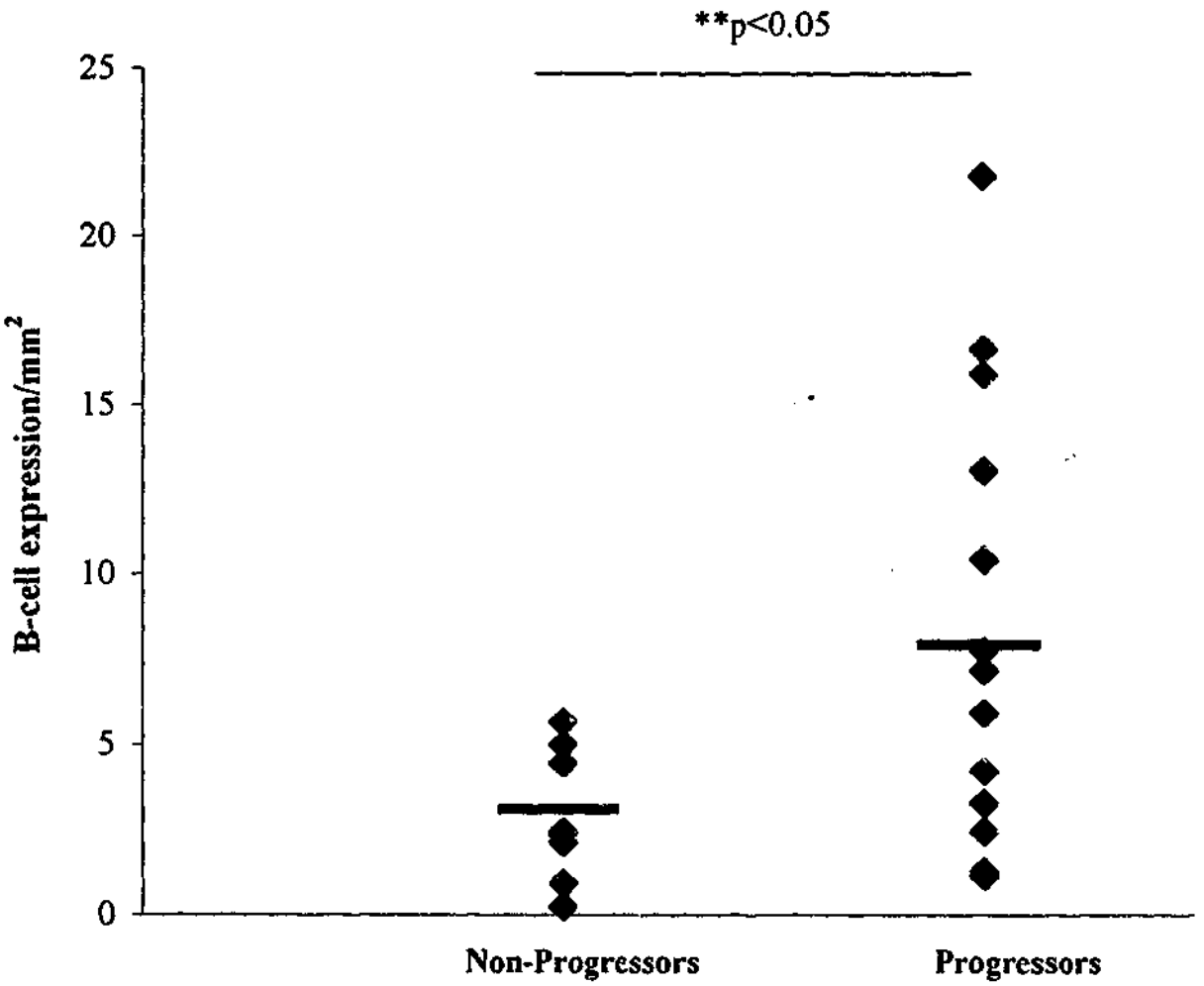

Figure 5.8 B-cell interstitial expression, non-progressors versus. progressors. Solid bars represent means. ** A significant difference between the two groups was noted with a p-value of <0.05.

5.8.3 Alpha Smooth Muscle Actin Expression

The intensity and distribution of α-SMA was noted within the biopsy samples. In particular, glomerular expression of α-SMA was distinguished from peri-glomerular expression and from interstitial expression. The intensity of expression within each compartment was graded from 0 to 3 as discussed in Section 5.4.

Mean glomerular and peri-glomerular expression of α-SMA was similar for patients with progressive and non-progressive disease, with no significant differences noted between the two groups. For patients with non-progressive disease mean glomerular scoring for α-SMA expression was 1.44 (SEM ±0.23) and mean peri-glomerular scoring was 1.55 (SEM ±0.23). For patients with progressive disease mean glomerular scoring for α-SMA expression was 1.72 (SEM ±0.17) and mean peri-glomerular scoring was 1.60 (SEM ±0.19).

The expression of interstitial α-SMA did differ between the two groups although not statistically significant ($p<0.055$). Patients with non-progressive disease had a mean score of 1.4 (SEM ±0.16) for interstitial α-SMA expression compared with 2.15 (SEM ±0.22) for patients with progressive disease. (Figure 5.9)

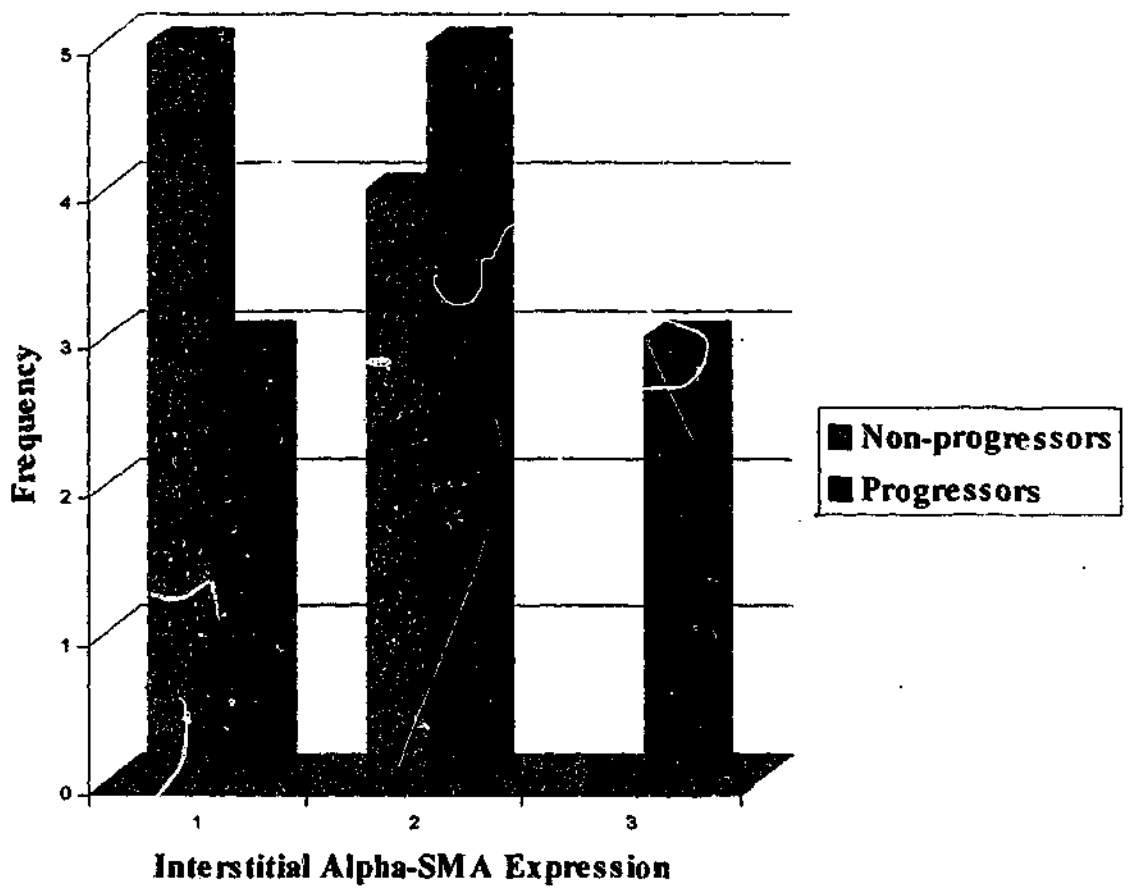

Figure 5.9 The expression of interstitial α-SMA, non-progressors versus progressors. Chart represents frequency of scores for each patient group. The difference between the two groups was not significant ($p<0.055$).

5.8.4 Expression of Regulators of Apoptosis

The final component of the immunohistochemical analysis of the biopsy samples examined the expression of some of the protein regulators of apoptosis in IgA nephropathy. Specific proteins examined included the pro-apoptotic molecule Fas-ligand and Bax and the anti-apoptotic molecules Bcl-2 and Bcl-X_L.

Where practical, the cells expressing the protein of interest were counted and expressed per mm^2. This was possible for measuring the expression of Bcl-2 and Bax. The expression of the other two proteins Fas-ligand and Bcl-X_L, were scored on a scale of 0 to 3 as described in Section 5.5. Tubular Bcl-2 expression, as opposed to cellular Bcl-2 expression was also scored from 0 to 3.

There was no *glomerular* expression noted of any of the apoptotic regulators in the biopsy samples from both patient groups. Within the tubulointerstitium however, Bcl-2 was noted to be expressed in association with infiltrating leucocytes. As well there was some tubular epithelial cell staining of Bcl-2 noted. Bax was similarly expressed in association with infiltrating tubulointerstitial leucocytes with no tubular expression noted. Fas-ligand and Bcl-X_L were only observed to be expressed on tubular epithelial cells.

The mean interstitial cellular expression of Bcl-2 in patients with non-progressive IgA nephropathy was 8.44 cells/mm^2 (SEM ±2.9) compared with 24.31 cells/mm^2 (SEM ±5.8) for patients with progressive disease. This represented a significant difference between the two patient groups ($p<0.04$). (Table 5.3 and Figure 5.10) Mean tubular expression scoring for Bcl-2 was 0.88 (SEM ±0.28) for non-progressors and 1.38 (SEM ±0.31) for progressors. This did not represent a significant difference. (Table 5.3)

Expression of the other apoptotic proteins Fas-ligand, Bax and Bcl-X_L was noted in both groups of patients. However no significant differences in their expression was observed between progressors and non-progressors. A trend to greater expression of Fas ligand in patients with non-progressive disease was noted ($p<0.10$). (Table 5.3)

Apoptotic Protein	Non-progressor Mean (±SEM) n=9	Progressor Mean (±SEM) n=13	P Value
Interstitial Bcl-2 cells/mm^2	8.44 (2.9)	24.31 (±5.8)	<0.04
Tubular Bcl-2 Score 0-3	0.88 (±0.28)	1.38 (±0.31)	NS
Fas-ligand Score 0-3	1.63 (±0.30)	0.85 (±0.29)	NS
Bax cells/mm^2	4.34 (±1.04)	4.22 (±1.10)	NS
Bcl-X_L Score 0-3	1.75 (±0.34)	1.33 (±0.22)	NS

Table 5.3 Apoptotic protein expression; non-progressors versus progressors.

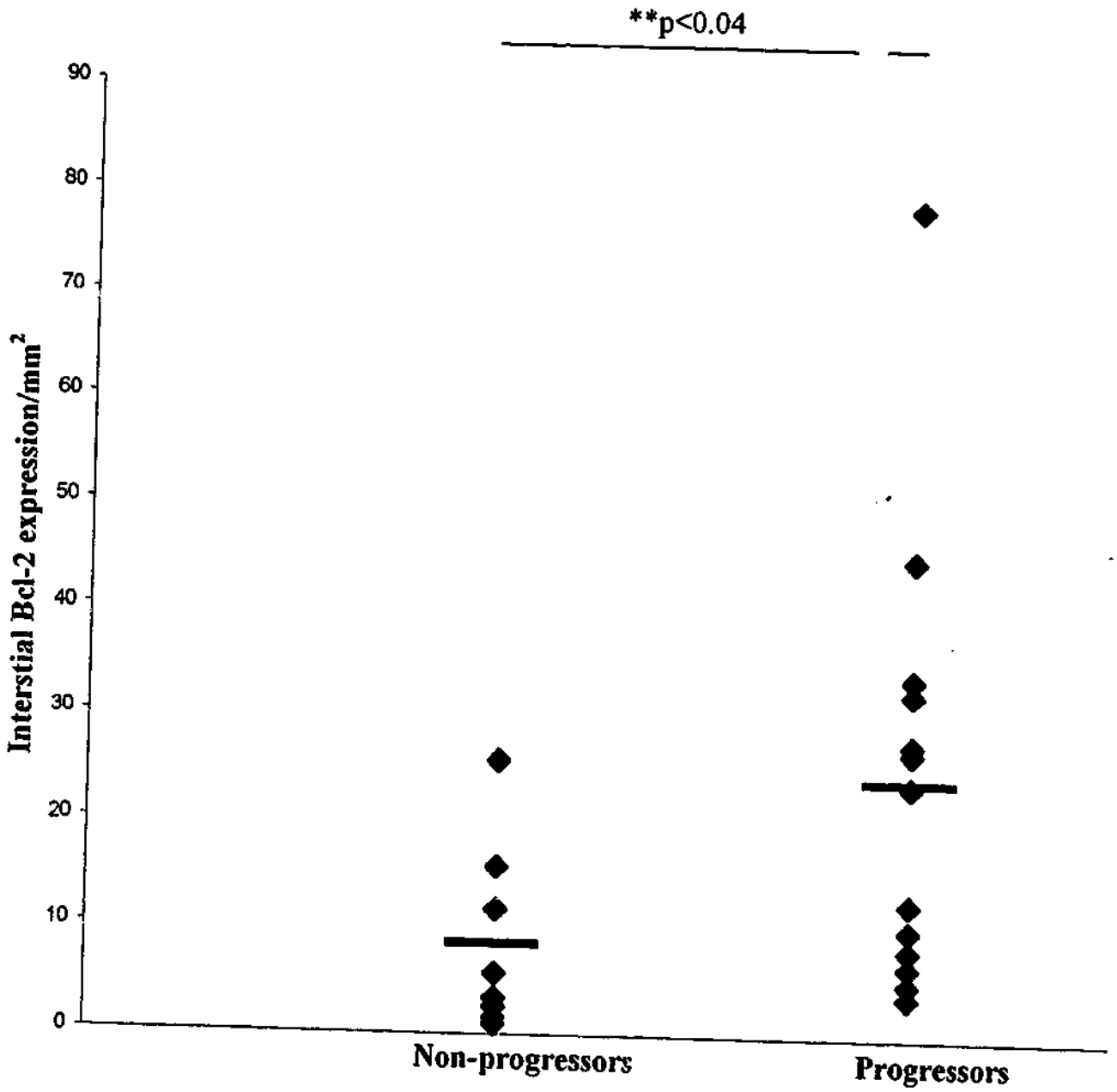

Figure 5.10 Interstitial Bcl-2 expression, non-progressors versus progressors. Solid bars represent means. ** A significant difference between the two groups was noted with a p-value of <0.04.

5.8.5 Significant Associations with Glomerular Infiltrate

Whilst there was no significant association between glomerular macrophage infiltration and disease progression, there was an association between glomerular macrophage number and α-SMA expression. Glomerular α-SMA expression correlated strongly with glomerular macrophage number ($p<0.01$), as did peri-glomerular α-SMA expression ($p<0.02$). Tubulointerstitial expression of the pro-apoptotic protein Bax correlated with glomerular macrophage number ($p<0.01$), as did interstitial Bcl-2 expression ($p<0.05$). (Table 5.4)

Correlating with Glomerular Macrophage Number	p value
Glomerular α-SMA expression	<0.01
Peri-glomerular α-SMA expression	<0.02
Bax expression	<0.01
Bcl-2 expression	<0.05

Table 5.4 Significant correlations with glomerular macrophage infiltration.

5.8.6 Significant Associations with Interstitial Infiltrate

The total number of infiltrating leucocytes was found to correlate strongly with the expression of Bcl-2 within the interstitium ($p<0.002$). Not surprisingly, interstitial T-cell numbers also correlated strongly with Bcl-2 expression. This was to be expected given that T-cells were the predominant infiltrating leucocytes for both patient groups. A similar correlation was noted between interstitial B-cell numbers and Bcl-2 expression as well as interstitial macrophage number and Bcl-2 expression. What was not anticipated was that the intensity of T-cell infiltration within the interstitium correlated with the degree of proteinuria at the time of renal biopsy, ($p<0.02$). (Table 5.5)

Cell Type	Variable	p value
Total leucocytes	Bcl-2	<0.002
T-cell	Bcl-2	<0.001
T-cell	Proteinuria	<0.02
B-cell	Bcl-2	<0.005
Macrophage	Bcl-2	<0.0005

Table 5.5 Significant correlations with infiltrating leucocytes.

5.8.7 Significant Associations with α-SMA Expression

As mentioned there was an association between glomerular α-SMA expression and glomerular macrophage number. Glomerular α-SMA expression was also strongly linked to tubular Bcl-2 expression ($p<0.001$) as well as Bcl-X_L expression ($p<0.02$). As anticipated, the renal biopsy sclerosing injury score correlated with interstitial α-SMA ($p<0.01$). Interstitial α-SMA expression also correlated with the total interstitial leucocyte cell count ($p<0.01$). (Table 5.6)

Significant Correlations with α-SMA Expression	p value
Sclerosing injury with interstitial α-SMA	<0.01
Tubular Bcl-2 expression with glomerular α-SMA	<0.001
Bcl-X_L expression with glomerular and interstitial α-SMA	<0.02
Interstitial leucocyte number and interstitial α-SMA	<0.01

Table 5.6 Significant correlations with α-SMA expression.

5.8.5 Significant Associations with Apoptotic Proteins

Of note there was a highly significant correlation between Bcl-2 expression and the degree of proteinuria at the time of biopsy ($p<0.004$). As well, the expression of Bcl-

2 was significantly associated with the sclerosing injury score, ($p<0.05$). This may well reflect the already established relationship between Bcl-2 expression and disease progression. (Table 5.7)

It was surprising to the candidate that no significant correlations were observed in association with the expression of the pro-apoptotic proteins Fas-ligand or Bax. As mentioned, there was a trend towards non-progressors having increased Fas-ligand expression compared with progressors ($p<0.10$).

Significant Correlations with Apoptotic Proteins	p value
Proteinuria and Bcl-2 expression	<0.005
Sclerosing injury and Bcl-2 expression	<0.05

Table 5.7 Significant correlations with apoptotic proteins.

5.9 Discussion

In this section of the thesis, observations have been made on a variety of aspects relating to the inflammatory and fibrotic processes involved in the progression of IgA nephropathy.

Firstly, the cells involved in glomerular inflammation in both progressive and non-progressive IgA nephropathy were identified as being predominantly macrophage and polymorph in origin. There were overall low number of inflammatory cells per glomeruli identified in both patient groups. Of note, the degree of glomerular inflammatory infiltrate did not differ between patients with progressive and non-progressive disease.

These observations concur with other studies already discussed, demonstrating glomerular hypercellularity in IgA nephropathy is explained largely by proliferation of resident mesangial cells rather than by infiltrating leucocytes [22, 179].

Of note, the degree of glomerular infiltration correlated with the expression of glomerular and peri-glomerular α-SMA. Other authors have made similar observations in IgA nephropathy [183] suggesting a relationship between macrophage infiltration in

particular and the expression of α-SMA. As the increased expression of α-SMA in IgA nephropathy and other proliferative glomerulonephritidies is thought to be due to activation of mesangial cells and their phenotypic transformation, it is possible that this is mediated via infiltrating inflammatory cells and their expression of various cytokines. As normal renal tissue expresses very little α-SMA [182], it is likely that disease-specific factors are responsible for this phenotypic transformation of mesangial cells.

The predominant infiltrating tubulointerstitial cells observed in the biopsies were T-cells. Lesser numbers of B-cells and macrophages were also present. The increased number of infiltrating T-cells observed in patients with progressive disease was not found to be statistically significant. In contrast the increased number of tubulointerstitial B-cells observed in patients with progressive disease was found to be statistically significant. As mentioned other authors have also found that T-cells, in particular $CD4^+$ cells are the dominant infiltrating cell type [30]. To the candidate's knowledge, the increased tubulointerstitial B-cell number in patients with progressive disease has not previously been observed. Alexopoulos et al [30] has observed however that patients with significant numbers of tubulointerstitial B-cells had a more rapid decline in renal function than those without. This suggests that the presence of tubulointerstitial B-cells may be marker of more severe tubulointerstitial injury or reflect an alternative cytokine profile linked to disease progression.

Of note the number of tubulointerstitial T-cells correlated strongly with the degree of proteinuria at biopsy. As proteinuria has been established as a surrogate marker of disease progression, this suggests a more significant relationship between disease progression and tubulointerstitial T-cell number than was observed. As has been suggested, the toxicity of filtered proteins to tubular cells is likely to result in tubular apoptosis and atrophy, with secondary interstitial infiltration of inflammatory cells. This mechanism of tubulointerstitial injury would explain the relationship between the degree of proteinuria and tubulointerstitial T-cell infiltration.

All of the regulators of apoptosis that were examined were identified in the renal biopsy specimens. Patients with both progressive and non-progressive IgA nephropathy expressed significant amounts of Bcl-2, Bax, Fas-ligand and Bcl-X_L. Of note Bcl-2 and Bax were expressed mainly in association with infiltrating interstitial cells with the other proteins being expressed on tubular epithelial cells.

In patients with progressive disease the number of tubulointerstitial cells expressing Bcl-2 was significantly greater compared to patients with non-progressive disease. This was not surprising in view of the fact that Bcl-2 expression correlated so strongly with the interstitial inflammatory infiltrate. Bcl-2 expression correlated significantly with all sub-types of inflammatory cells examined including T-cells, B-cells and macrophages.

It is thus clear that there is an association between Bcl-2 expression and the intense tubulointerstitial infiltrate observed in patients with progressive IgA nephropathy. Bcl-2 is likely to play a role in the propagation of the interstitial infiltrate, particularly protecting inflammatory cells against apoptosis. As the tubulointerstitium of patients with progressive disease is characterised by fibrosis and atrophy, cell survival may be challenged due to ischaemia or a deficiency of intercellular adhesion molecules. The expression of Bcl-2 may therefore protect these vulnerable infiltrating cells from undergoing apoptosis. It is possible that interstitial Bcl-2 expression is thus secondary to the disease process itself, rather than relating to the overall degree of interstitial injury.

Fas-ligand was noted to be expressed on tubular epithelial cells and the expression of this potent stimulus for apoptosis was increased in patients with progressive disease, although this difference was not statistically significant ($p<0.10$). As has already been discussed, it is likely that proteinuria is directly or indirectly toxic to the tubular epithelial cells, inducing apoptosis. As progressive IgA nephropathy is associated with significantly increased proteinuria, induction of tubular Fas-ligand expression may play a role in the development of tubular atrophy characteristic of progressive disease.

In this chapter the candidate has described the pattern of expression of four regulators of apoptosis in IgA nephropathy but it has not been possible to accurately quantify their expression. In the next two chapters, the expression of mRNA for Bax and Bcl-2 has been quantitated by competitive PCR and the expression of mRNA for Fas and Fas-ligand has been quantitated by real-time PCR.

CHAPTER SIX

THE EXPRESSION OF BAX AND BCL-2 mRNA IN IGA NEPHROPATHY BY COMPETITIVE PCR

6.1 Introduction

In the previous chapter, the candidate demonstrated by means of immunohistochemistry that there is clear expression of the two apoptotic regulatory proteins Bcl-2 and Bax in patients with progressive and non-progressive IgA nephropathy. It was observed that the interstitial expression of the anti-apoptotic molecule Bcl-2 correlated strongly with interstitial T and B cell infiltration as well as interstitial macrophage number. There was a corresponding relationship between Bcl-2 expression and disease progression, as well as with the already established surrogate markers of disease progression, proteinuria and biopsy sclerosing injury score. The expression of the pro-apoptotic molecule Bax significantly correlated to glomerular macrophage number but not with disease progression. In the interstitium, Bax and Bcl-2 were observed in association with infiltrating lymphocytes while Bax alone was also noted to be expressed on tubular epithelial cells.

It is clear that when trying to better understand the relevance of the expression of the various proteins that control the process of apoptosis, it would be best to examine *in isolation* the various compartments within the kidney. In this chapter the candidate has examined the expression of mRNA for Bax and Bcl-2 in IgA nephropathy using RNA extracted from whole renal biopsy tissue. As only small fragments of renal biopsy tissue were made available for RNA extraction, it was not possible to separate cortical from medullary tissue. As the renal cortex is usually the target of diagnostic renal biopsies, it can be assumed that the fragments provided to the candidate were predominantly cortical in origin. To prove this histological analysis of the biopsy fragments would have had to be performed, leaving very little tissue available for RNA extraction. This again highlights the limitations of analysing human renal biopsy samples.

RNA extracted from the cortex of normal nephrectomy tissue was used as controls. The mRNA expression of Bax and Bcl-2 was measured using the competitive PCR technique outlined in Section 2.6.

6.2 Patient Details

A total of 21 patients with IgA nephropathy had RNA available from renal biopsies for analysis. Seven normal samples from nephrectomies were used as controls. Thirteen patients had non-progressive IgA nephropathy as defined in Section 2.8.5. Eight patients had progressive IgA nephropathy. RNA was available from only these 21 patients with IgA nephropathy, as the practice of freezing spare renal biopsy tissue for later RNA extraction only commenced in the candidate's laboratory in 1994.

Of the thirteen patients with non-progressive disease analysed in this chapter, only one patient had been analysed in Chapter 4 for the expression of TUNEL positive cells and only three had been analysed in Chapter 5 for cellular infiltrates and the expression of apoptotic regulators. From the eight patients with progressive disease analysed in this chapter, one had been previously analysed in Chapter 4 and one in Chapter 5. Whilst it would have been helpful to analyse the same groups of patients for all sections of the thesis, this was not possible due to insufficient biopsy material. The normal controls used in this section of analysis included samples used as controls in Chapter 4.

Patients with non-progressive IgA nephropathy had a significantly lower mean age of 38 years (SEM ±5.2) compared to 52 years (SEM ±5.5) for patients with progressive disease ($p<0.01$). In both groups males were more common than females with a ratio of 1.6:1 in patients with non-progressive disease and 2:1 in patients with progressive disease. As anticipated patients with non-progressive disease had significantly lower mean serum creatinine concentrations and less proteinuria compared with patients with progressive disease. Sclerosing injury scores were similarly significantly lower in patients with non-progressive disease compared to patients with progressive disease. Proliferative injury scores were similar for the two groups of patients. (Table 6.1)

Clinical and Biopsy Characteristics	Non-Progressors Mean (±SEM)	Progressors Mean (±SEM)	P value
Age	38 (±5.2)	52 (±5.5)	<0.01
Sex (M:F)	1.6:1	2:1	NS
Serum Creatinine (μmol/L)	88 (±4.4)	296 (±59.8)	<0.0001
Proteinuria (g/24hrs)	1.2 (±0.5)	2.5 (±0.7)	<0.03
Sclerosing Injury Score	4.1 (±0.6)	7.9 (±0.4)	<0.02
Proliferative Injury Score	2.4 (±0.3)	3.4 (±1.0)	NS

Table 6.1 Details of two patient groups used in Bcl-2 and Bax analysis.

6.3 Statistical Analysis

Statistical analysis of the competitive PCR results was performed using SAS V 8.0 (SAS Institute Inc, Cary, NC, USA). Significance between continuous, non-normally distributed data was ascertained using Spearman correlation coefficients. A p-value of < 0.05 was considered to be statistically significant.

Post-hoc comparisons between the three groups examined were then performed using a Wilcoxon two-sample t-test. To account for multiple comparisons between the groups, a p value of < 0.02 was considered to be significant.

6.4 Results

6.4.1 Bcl-2 Expression

Bcl-2 mRNA was detected in small amounts in the 7 normal nephrectomy samples with a mean expression of 86 copies per ng of RNA (SEM ±33). Patients with IgA nephropathy, both progressors and non-progressors had a significantly higher mean Bcl-2 mRNA expression of 280 copies per ng RNA (SEM ±51) ($p<0.005$). (Figure 6.1)

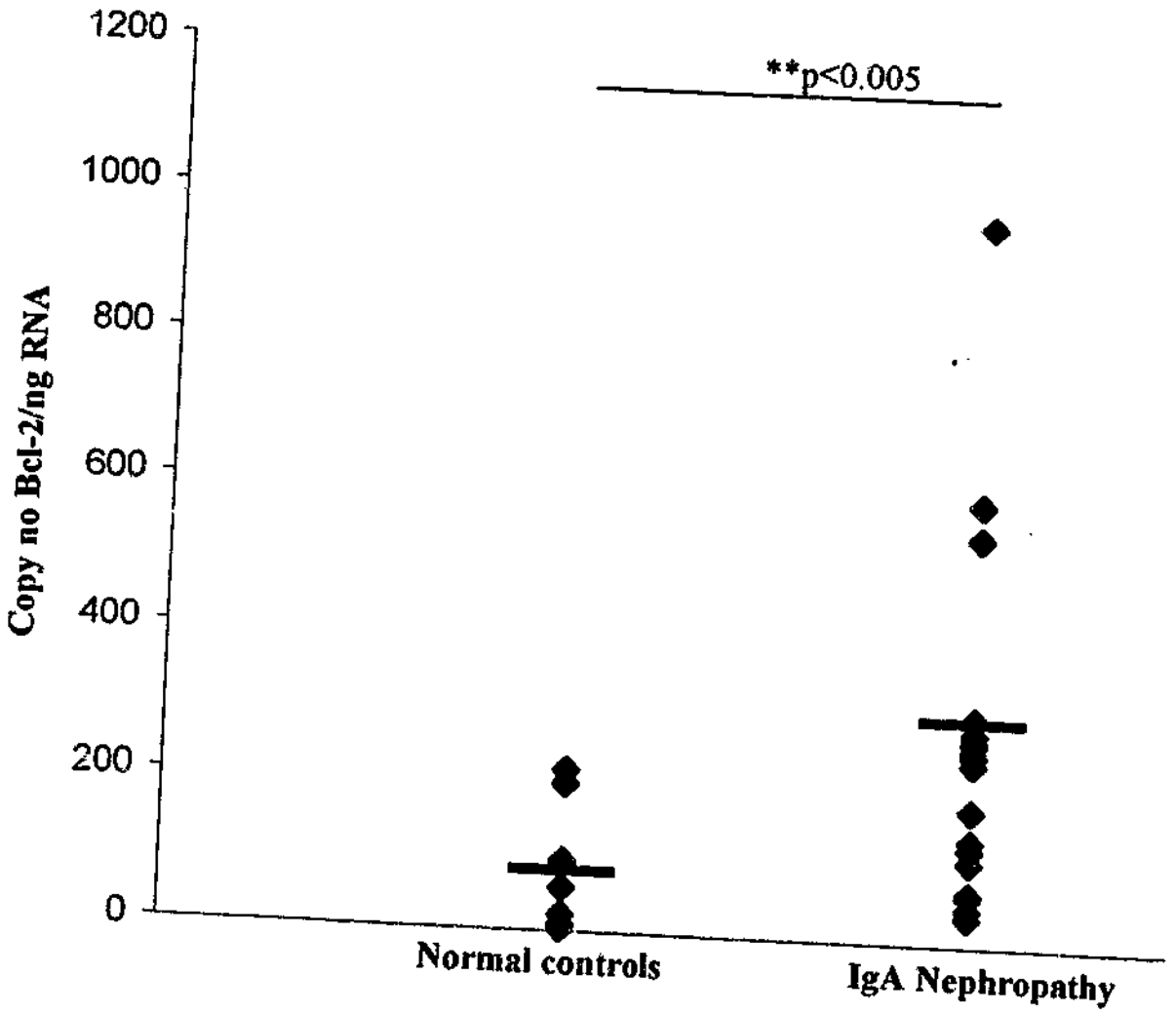

Figure 6.1 Bcl-2 expression, normal controls versus IgA nephropathy. Solid bars represent means. ** A significant difference was observed ($p<0.005$).

Patients with non-progressive IgA nephropathy had a mean Bcl-2 mRNA copy number of 204 per ng of RNA (SEM ±45). (Table 6.2). This was significantly greater than normal controls ($p<0.02$). Biopsy samples from patients with progressive IgA nephropathy had a mean expression of Bcl-2 mRNA of 283 copies per ng of RNA (SEM ±45). (Table 6.2) This was not significantly greater than patients with non-progressive disease but was significantly more than normal nephrectomy samples ($p<0.002$). (Figure 6.2)

Patient Group	Mean copy number Bcl-2/ng RNA (±SEM)
Normal (n=7)	86 (±33)
Non-Progressors (n=13)	204 (±45)
Progressors (n=8)	283 (±94)

Table 6.2 Mean expression of Bcl-2 in biopsy samples for the three patient groups. No significant difference was noted between non-progressors and progressors.

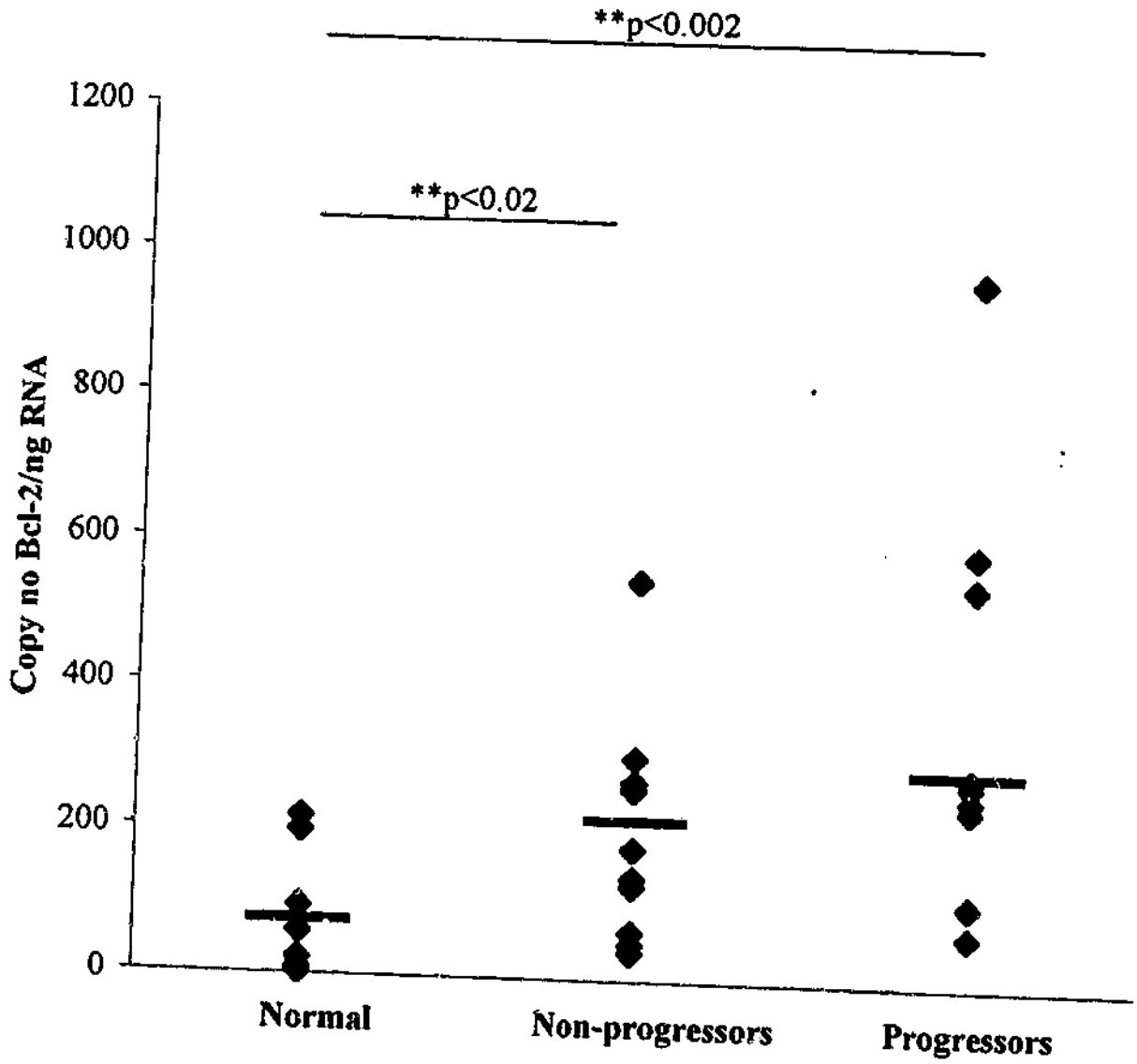

Figure 6.2 Bcl-2 expression, normal versus progressors versus non-progressors. Solid bars represent means. ** A significant difference in Bcl-2 expression was noted between normal and non-progressors and between normal and progressors.

6.4.2 Bax Expression

For the 7 normal control samples, the mean copy number of Bax mRNA was 6.78 per ng of RNA (SEM ±2.07). Patients with IgA nephropathy, both non-progressors and progressors had a mean Bax mRNA expression of 19.2 copies per ng RNA (SEM ±5.05). This did not represent a significant difference. (Figure 6.3)

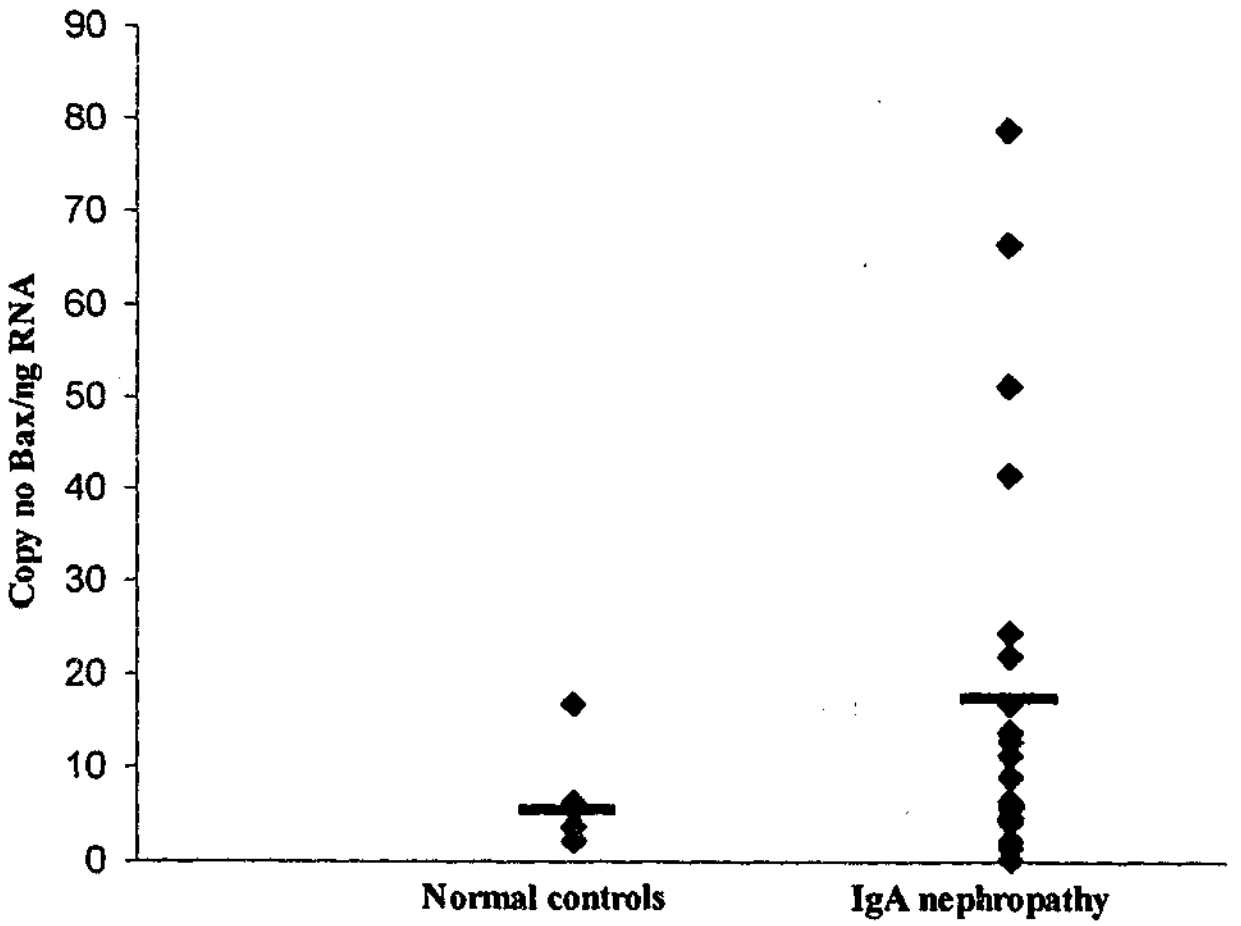

Figure 6.3 Bax expression, normal controls versus IgA nephropathy. Solid bars represent means. No significant difference was noted.

Patients with non-progressive disease had a mean Bax mRNA expression of 8.02 copies per ng RNA (SEM ±1.93). This was not significantly different from normal controls. The expression of Bax mRNA was significantly greater in patients with progressive IgA nephropathy compared to non-progressors with a mean copy number of 32.9 per ng RNA (SEM ± 9.30) (p value of <0.02). (Table 6.3 and Figure 6.4)

Patient Group	Mean copy number Bax/ng RNA (±SEM)
Normal (n=7)	6.78 (±2.07)
Non-Progressors (n=13)	8.02 (±1.93)**
Progressors (n=8)	32.9 (±9.30)**

Table 6.3 Mean expression of Bax in biopsy samples for the three patient groups. ** A significant difference was noted between non-progressors and progressors.

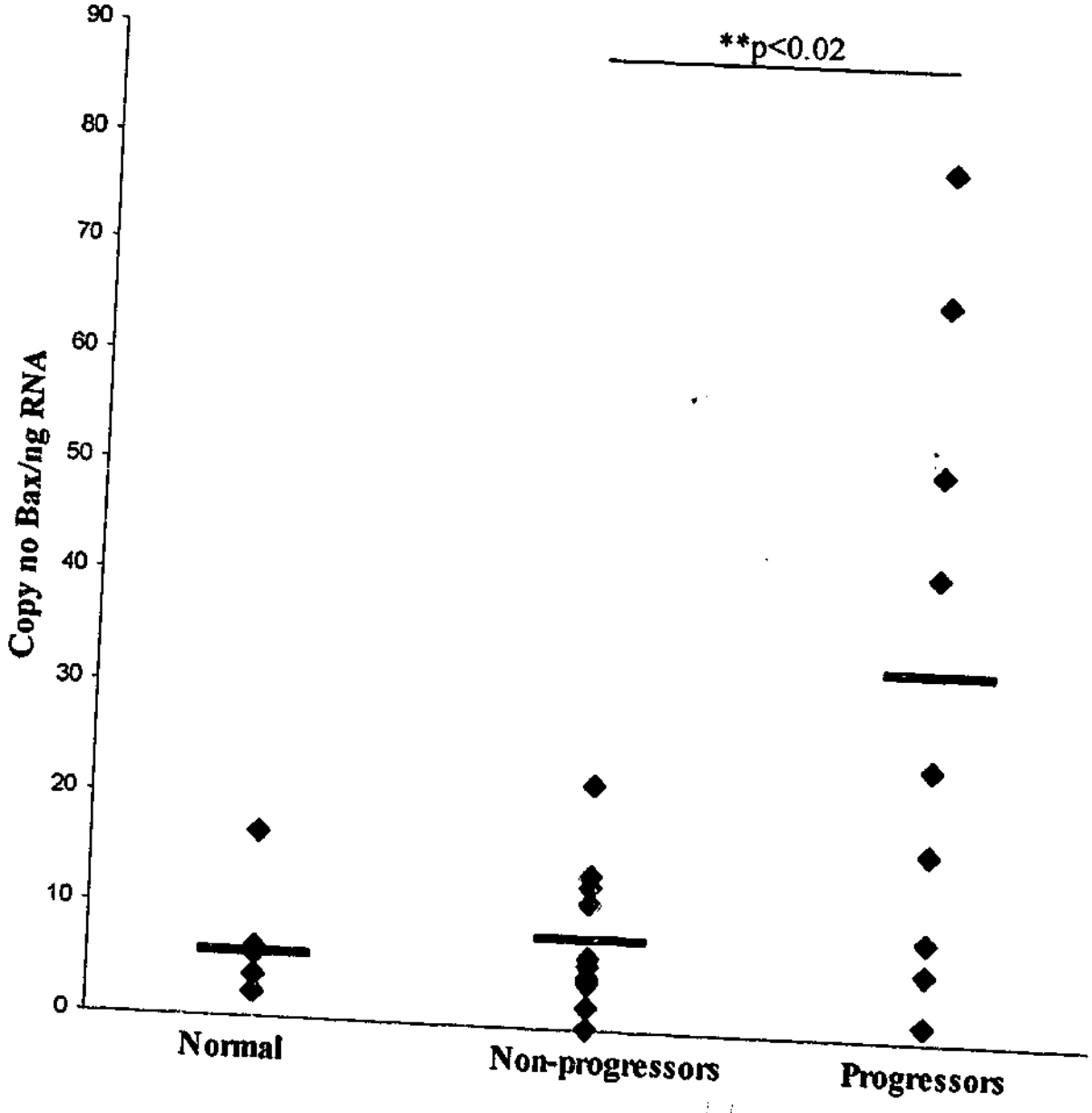

Figure 6.4 Bax mRNA expression, normal versus progressors versus non-progressors. Solid bars represent means. ** A significant difference in Bax expression was noted between non-progressors and progressors.

6.4.3 Significant Correlations with Bcl-2 mRNA Expression

Further analysis was performed to assess the relationship if any, between Bcl-2 mRNA expression and the measured clinical and histological characteristics for both patient groups. The relationship between Bcl-2 expression and disease progression, patient age, sex, serum creatinine, protein excretion, proliferative and sclerosing injury score was assessed.

As mentioned, there was a strong relationship between disease progression and Bcl-2 expression ($p<0.002$). The only other significant correlation noted was between Bcl-2 expression and Bax expression ($p<0.001$). No association was noted between Bcl-2 levels and the other parameters examined. This was somewhat surprising, as the candidate has already established that sclerosing injury and proteinuria are independent predictors of disease progression. In this instance, whilst Bcl-2 mRNA expression correlated with the progression of disease it did not shown a similar relationship with the surrogate markers for disease progression; sclerosing injury and proteinuria.

6.4.4 Significant Correlations with Bax mRNA Expression

Similar observations were noted when further analysis was performed of Bax expression and the clinical and histological patient characteristics. Whilst Bax levels correlated significantly with disease progression ($p<0.02$), there was no correlation with the other markers of disease progression. The relationship between Bax expression and sclerosing injury score on biopsy was not significant ($p<0.06$). (It should be recalled that for this section of analysis a p value of <0.02 was considered significant)

6.5 Discussion

In this section of the thesis the expression of mRNA for the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax was measured using RNA extracted from whole biopsy samples from patients with IgA nephropathy using normal nephrectomy samples as controls.

The levels of Bcl-2 mRNA expressed in the total IgA nephropathy patient population were significantly higher than in normal renal tissue. On sub-analysis, both IgA nephropathy progressors and non-progressors had significantly higher levels of Bcl-2 mRNA expression compared with controls. There was increased Bcl-2 mRNA expressed in progressors versus non-progressors but this difference was not statistically significant. In chapter 5, on immunohistochemical analysis, it was noted that Bcl-2 was expressed mainly on infiltrating T-cells in the tubulointerstitium. In that analysis the increased expression of Bcl-2 in progressors compared to non-progressors *was* found to be statistically significant.

It should be noted that the patients studied in Chapter 5 were not identical to those studied in Chapter 6. Although the two groups were similar in character both clinically and histologically with some overlap they were not identical. This may explain the apparent discrepancy between the results of immunohistochemical analysis of Bcl-2 levels compared to mRNA analysis of Bcl-2. However it is reassuring that there was a trend for increased Bcl-2 mRNA expression in patient with progressive disease.

A further possible explanation for the failure to demonstrate significantly increased Bcl-2 mRNA in progressors versus non-progressors is the focal nature of the increased expression of Bcl-2 as demonstrated by immunohistochemistry. Analysis of RNA from whole biopsy tissue may have therefore "diluted out" this increased expression. Analysis of mRNA by *in-situ* hybridisation may resolve this issue.

Although Bcl-2 mRNA expression was not significantly different between progressors and non-progressors, there was a significant correlation between disease progression and expression of Bcl-2. This correlation takes into account the differences observed between progressors and non-progressors as well as between progressors and controls. This once again supports the observations made in Chapter 5.

The expression of Bax mRNA was not found to be significantly increased in patients with IgA nephropathy compared to controls. However there was significantly increased Bax mRNA expression in progressors versus non-progressors. As shown in Chapter 5, Bax protein expression on immunohistochemistry was associated with tubular epithelial cells and interstitial inflammatory cells. There was no glomerular expression of Bax protein noted. However there was *no* increased expression of Bax protein in progressors compared to non-progressors noted in that analysis. It must be recalled that only a crude scoring system was utilized for measuring expression of Bax protein with the immunohistochemical analysis. It is possible that this method was not sensitive enough to detect a real difference in Bax protein expression. Moreover as mentioned, the patient groups for the two sections of analysis were not identical and thus the difference in results may be explained by the different biopsy samples examined in the two sections.

It is also possible that the discrepancy noted between Bax mRNA expression by competitive PCR and Bax protein expression by immunohistochemistry represents a real difference that arises due to factors that influences the translation of Bax mRNA into Bax protein. Whilst Bax mRNA expression may be significantly increased in patients with progressive IgA nephropathy, there may be factors that limit or reduce translation of Bax mRNA into Bax protein such that there is no detectable difference in protein expression between the two patient groups. For example Pepper et al [187] have demonstrated *in vitro* that Bax antisense oligonucleotides can inhibit Bax translation leading to down regulation of Bax protein expression and the inhibition of apoptotic cell death. Post-translational cleavage of apoptotic proteins by endogenous proteases can also reduce their activity as demonstrated by Lin et al [188] for Bcl-2. The shortened fragment produced by protein cleavage may not be detectable by immunohistochemistry depending on the site of protein cleavage and the specific antibody epitope. It is therefore possible that such factors have resulted in no detectable difference in Bax protein expression between the two patient groups.

Of note there was a strong association between Bcl-2 mRNA expression and Bax expression as measured by competitive PCR. This suggests a relationship between the two proteins even though their effect on cell survival are conflicting. As has been discussed these two molecules form heterodimers to exert their effect on cell survival.

The balance in expression of pro-apoptotic versus anti-apoptotic members of the Bcl-2 family of proteins decides the fate of a cell. Co-expression of the two molecules with-in the interstitium would explain the correlation in their measured expression, in particular in association with infiltrating inflammatory cells.

As discussed, there have been several studies that have examined the distribution of Bax and Bcl-2 in human glomerulonephritis. Most of these have concentrated on the glomerulus with very few observations being made on the tubulointerstitium [150, 152]. Nakopoulou et al [151] did note Bcl-2 expression in the proximal convoluted tubules in biopsies from patients with a variety of glomerulonephritidies. They did not relate this to disease progression or any other clinical or histological parameters.

The observations made by the candidate are therefore unique and in combination with the observations made on the distribution of Bax and Bcl-2 protein in the tubulointerstitium give some insight into the pathogenesis of tubulointerstitial injury in progressive IgA nephropathy. As has already been discussed, in almost all forms of glomerulonephritis, it is the degree of tubulointerstitial damage that is most closely linked to irreversible renal injury.

CHAPTER SEVEN

THE EXPRESSION OF FAS AND FAS LIGAND mRNA IN IGA NEPHROPATHY BY REAL TIME PCR

7.1 Introduction

In this chapter the expression of mRNA for Fas and Fas-ligand in IgA nephropathy has been determined by real-time PCR analysis. The group of patients studied were the same as those studied in Chapter 6 for the expression of Bax and Bcl-2.

7.2 Real-Time PCR Validation Experimentation

As the candidate was unable to establish reliable standard curves for Fas and Fas ligand using competitive PCR, it was decided to attempt to measure the expression of these genes using the relatively new technology of real-time PCR. The laboratory had recently acquired an ABI PRISM® 7700 Sequence Detector System. Using pre-developed assay reagents (PDAR) purchased from Applied Biosystems, the expression of Fas and Fas ligand mRNA was measured in the extracted renal tissues. The PDARs had been pre-optimised by the manufacturing company when utilized with their Universal PCR Master Mix and thus optimisation experiments were not necessary.

As outlined in the methodology section of the thesis, separate-well or multiplex reactions are possible with real-time PCR. The initial experiments examining the expression of Fas were done by the separate-well technique outlined in Section 2.7.4.4. Once the candidate was familiar with the technology, multiplex reactions were performed for expression of Fas-ligand. These reactions utilised considerably less reagents and were easier to prepare and thus preferred to the separate-well technique. (See Section 2.7.4.5)

There are two methods available for interpretation of real-time PCR results. One involves setting up standard curves for the target gene and endogenous control using known dilutions of cDNA; much like the methodology used by the candidate in

competitive PCR. The other uses arithmetic formulae to directly compare target and endogenous controls in each sample. This process, known as relative quantitation, was tested by the candidate without success. The failed validation experiments are shown below and are discussed in detail as they do reveal a potential flaw in real-time PCR applications.

7.2.1 Relative Quantitation Technique for Real-Time PCR

The relative quantitation technique involves the use of arithmetic formulae to directly compare target gene expression with endogenous control expression. This technique precludes the need to establish a standard curve as used in competitive PCR. This process relies on establishing the fact that both the target gene and the endogenous control are amplified at equal efficiencies by the real-time PCR reaction over the range of concentrations seen in the test samples. Only once this has been validated can one assume this relationship to be constant for each test sample and thus can compare target gene expression between each test sample. It must be recognised that the endogenous control, in the candidate's case 18S ribosomal RNA, is expressed in much greater abundance than the target gene and therefore equal efficiencies for the two reactions cannot be assumed.

The validation experiment involves the construction of two efficiency curves, one for the gene of interest and the other for the endogenous control. This charts the relationship between the delta Ct, the target gene Ct less the endogenous control Ct, over a range of dilutions of cDNA. If the reactions are occurring at equal efficiencies at all dilutions of cDNA, then the relationship between the target gene Ct and the endogenous control Ct should be constant for all dilutions. Furthermore the graph of delta Ct versus the log of the input concentration of cDNA should be a straight line. The accepted error margin for this graphed relationship is ±0.1, with a target gradient of zero.

Unfortunately, for reasons that are not clear, the candidate was unable to achieve an acceptable gradient for the validation experiment for the gene Fas and 18S using the purchased reagents. Figure 7.1 shows the graph of absolute Ct versus the log of the input concentration of cDNA for the target gene Fas and Figure 7.2 demonstrates this relationship for the endogenous control 18S. As can be seen the R^2 for both these graphs

are close to 1.00 confirming the linear relationship between the log input cDNA and absolute Ct for the separate reactions.

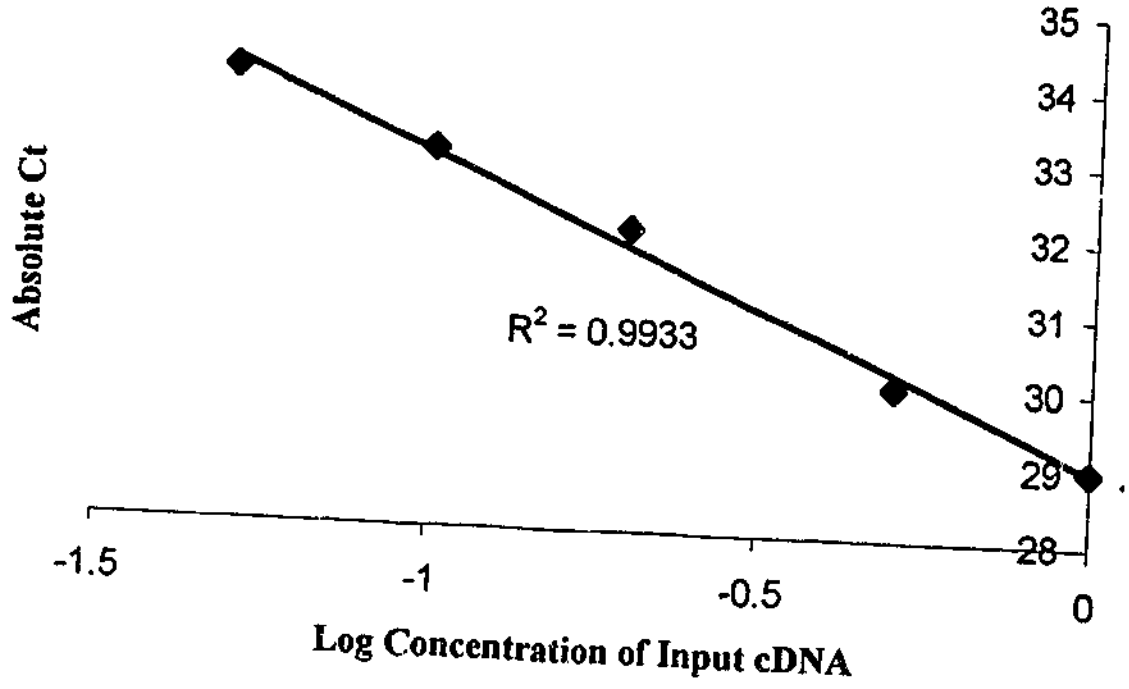

Figure 7.1 Graph of log concentration of input cDNA versus absolute Ct for Fas.

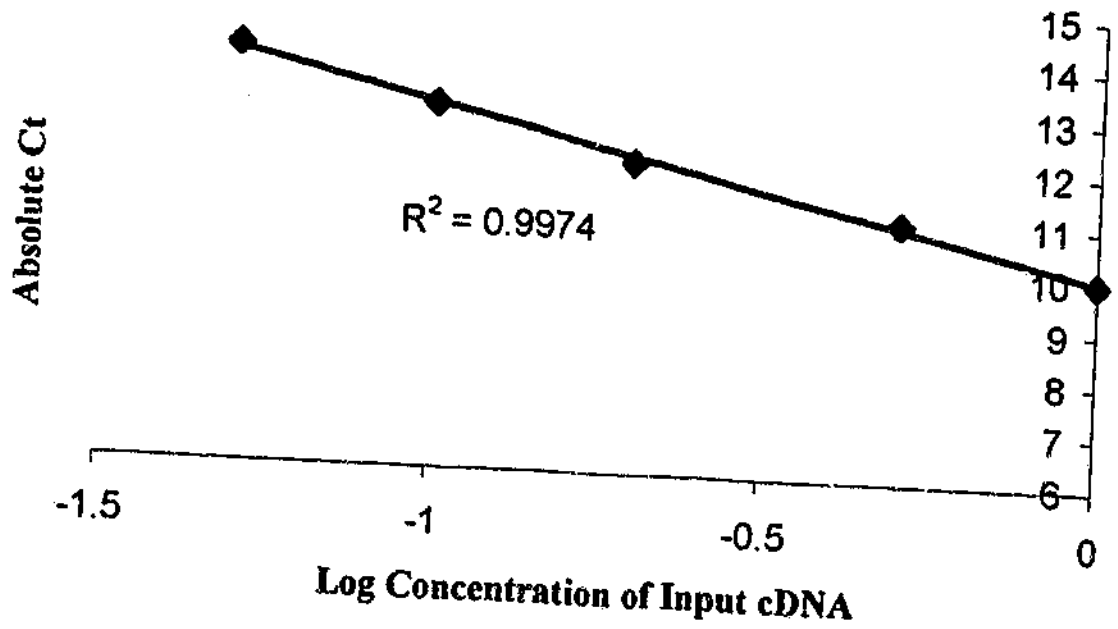

Figure 7.2 Graph of log concentration of input cDNA versus absolute Ct for 18S.

If the efficiencies of the two reactions are equal, when the two validation experiments are combined and a graph of delta Ct (absolute Ct of Fas less absolute Ct of 18S) and log concentration of input cDNA is constructed, a near straight line should result. This would confirm that despite the differing concentrations of target gene and endogenous control within the dilutions of the validation experiment, the efficiencies of the two reactions remain unchanged. The graph constructed from Figure 7.1 and 7.2 is shown in Figure 7.3.

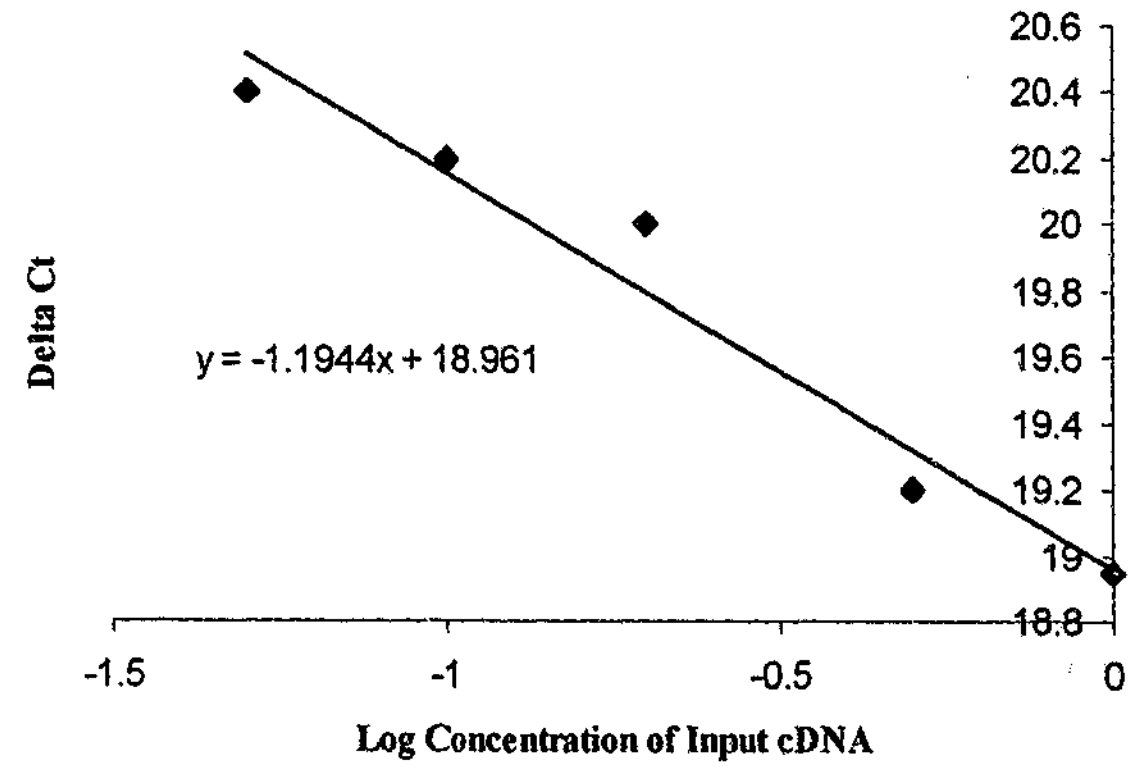

Figure 7.3 Graph of delta Ct versus log concentration of input cDNA.

Unfortunately, as can be seen from Figure 7.3, the efficiencies of the two reactions is not constant and the gradient of the trendline is – 1.19, much in excess of the accepted gradient error range of ±0.1. The experiment was repeated a number of times without achieving a gradient of less than ±0.25 and thus this methodology had to be abandoned.

The reason for this problem in achieving an acceptable validation experiment remains unclear. The explanation for this discrepancy probably lies in the disparity between the concentration of target gene and endogenous control. It is important to recall that a delta Ct of 20 represents an absolute difference in concentrations between the target gene and the endogenous control of 2^{20} given the exponential nature of the polymerase chain reaction. It is likely that the efficiency of the PCR reactions differ at these two concentrations of the PCR target. If an endogenous control could be identified that is expressed in a concentration range similar to the target gene it is more likely that the two reactions would take place at equivalent efficiencies. Due to the costs involved in testing a range of different endogenous controls to identify such a gene, it was decided by the candidate to revert to the standard curve methodology.

7.2.2 Standard Curve Quantitation Technique for Real-Time PCR

The standard curve methodology for quantifying real-time PCR results involves construction of two separate standard curves, one for the target gene and the other for the endogenous control over a range of known dilutions of cDNA. The expression of the target gene or the endogenous control in a test sample is then determined from the absolute Ct value of the sample relative to the appropriate standard.

Once the amount of target gene present in the test sample is determined, it is expressed relative to the amount of endogenous control present in the sample. This corrects for the differing amounts of cDNA and therefore RNA present in each sample. This final figure has no units (arbitrary units) and represents the amount of the gene of interest present within the test sample corrected for total cDNA.

The constructed standard curves for Fas, Fas ligand and 18S are shown in Figure 7.4. Note only a five point standard curve could be constructed for Fas ligand as any further dilutions of cDNA would result in a Ct value greater than the no template (negative) control. An R^2 value of greater 0.90 was deemed acceptable for the trendline.

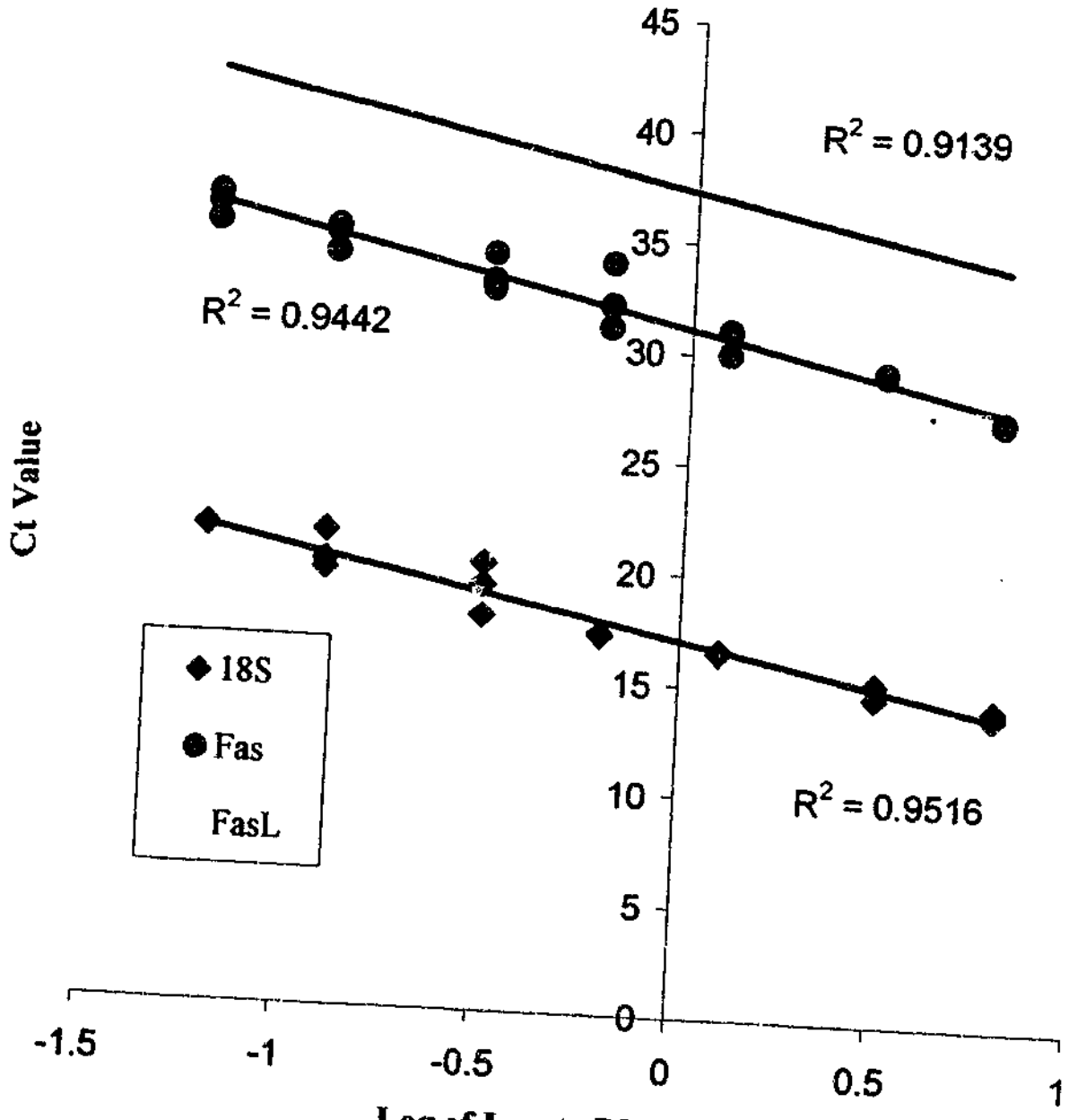

Figure 7.4 Standard curves constructed for Fas, Fas ligand and 18S for use in real time-PCR. An R^2 value of greater than 0.90 for the trendline was deemed acceptable.

7.3 Patient Details

The biopsy samples analysed with real-time PCR were the same as those used in the previous chapter for competitive PCR. In Chapter 6, Table 6.1 describes the patients' characteristics, clinical and histological parameters with comparisons between progressors and non-progressors. As for competitive PCR, real-time PCR analysis was performed using normal nephrectomy tissue as non-disease controls.

It should be noted that new RT (reverse transcriptase) had to be prepared from RNA extractions for real-time PCR analysis. Section 2.7.3 describes the rationale for the use of random hexamers rather than oligo-thymidilic acid for RT preparation in real-time PCR.

7.4 Statistical Analysis

Statistical analysis of the real-time PCR results was performed using SAS V 8.0 (SAS Institute Inc, Cary, NC, USA). Significance between continuous, non-normally distributed data was ascertained using Spearman correlation coefficients. A p-value of < 0.05 was considered to be statistically significant.

Post-hoc comparisons between the three groups examined were then performed using a Wilcoxon two-sample t-test. To account for multiple comparisons between the groups, a p value of < 0.02 was considered to be significant.

7.5 Results

7.5.1 Fas mRNA Expression by Real Time PCR

The amount of Fas expressed for the three patient groups is shown in Table 7.1. The mean amount of Fas expressed in the normal samples was 0.15 (SEM ±0.02). The mean amount of Fas expressed for all patients with IgA nephropathy, both non-progressors and progressors was 0.21 (SEM ±0.03). This did not represent a significant difference. (Figure 7.5)

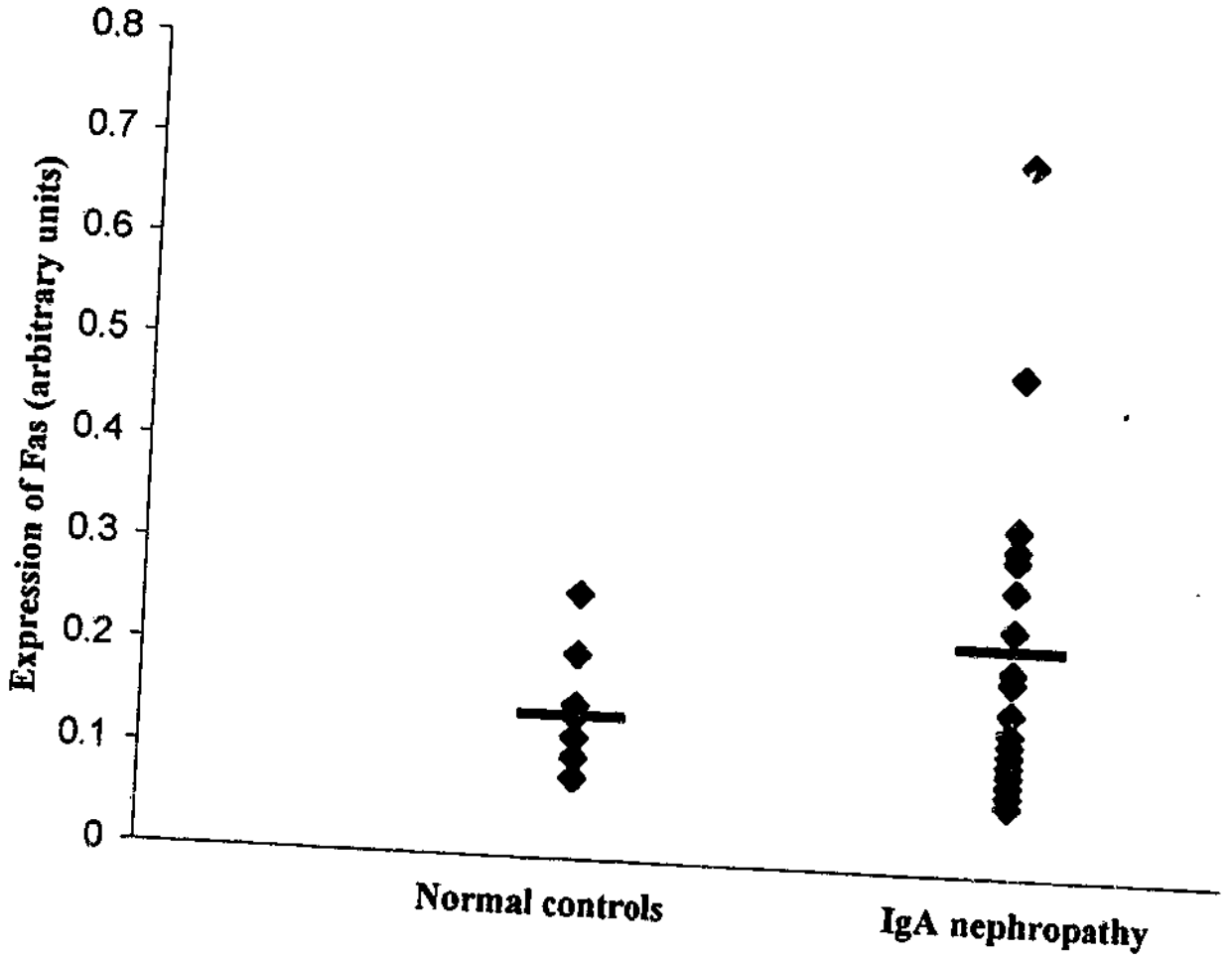

Figure 7.5 Expression of Fas mRNA, normal controls versus IgA nephropathy. Solid bars represent means. No significant difference was noted.

The mean amount of Fas expressed in biopsies from patients with non-progressive disease was 0.16 (SEM ±0.04), compared to a mean of 0.28 (SEM ±0.06) for patients with progressive disease. (Table 7.1). This represented a significant difference ($p<0.02$). The difference between expression of Fas in normal controls and patients with non-progressive disease was not significant. Similarly the difference between the expression of Fas in normal controls and patients with progressive disease was also not found to be statistically significant ($p<0.04$). (Figure 7.6)

Patient Group	**Expression of Fas mRNA Mean (±SEM) (arbitrary units)**
Normal (n=7)	0.15 (±0.02)
Non-Progressors (n=13)	0.16 (±0.04)**
Progressors (n=8)	0.28 (±0.06)**

Table 7.1 Mean expression of Fas mRNA for the three patient groups. ** A significant difference was noted between non-progressors and progressors.

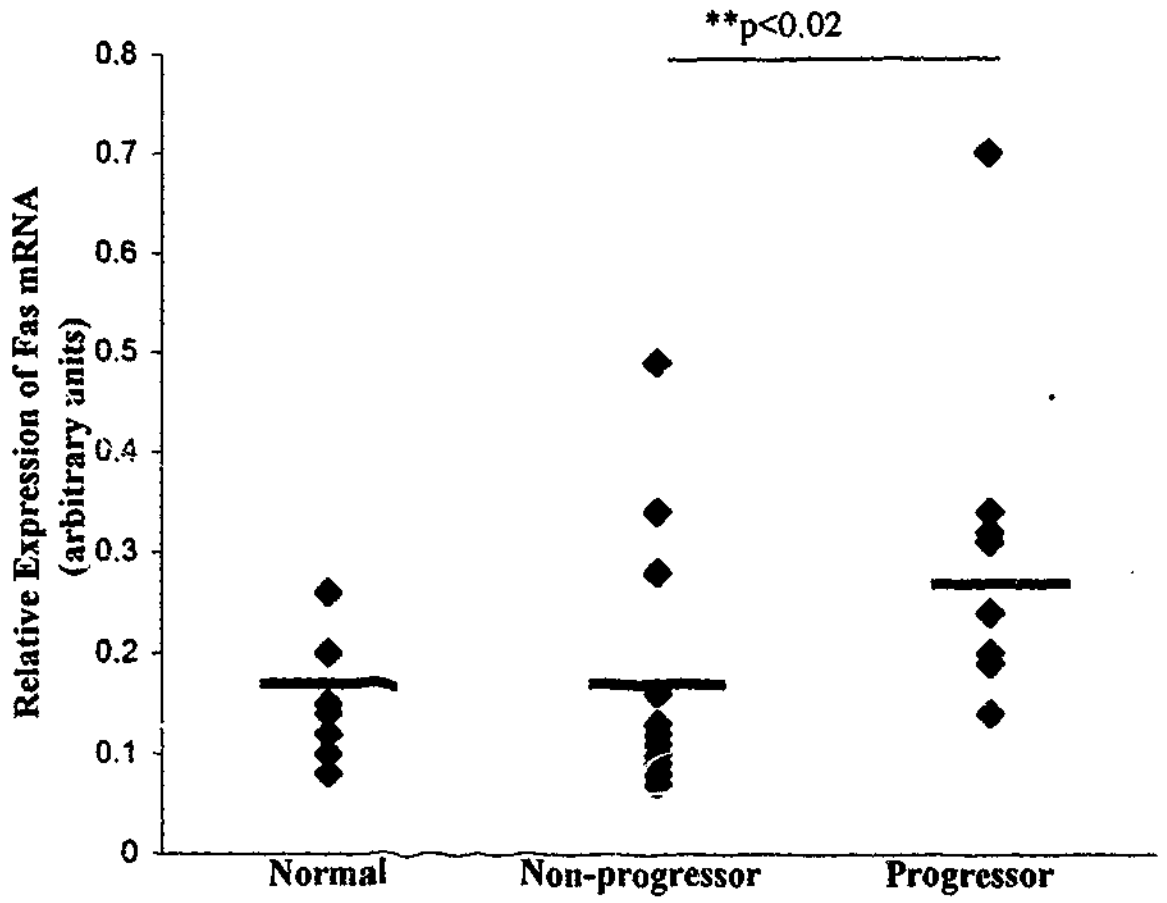

Figure 7.6 Fas expression, normal versus progressors versus non-progressors. Solid bars represent means. ** A significant difference in Fas expression was noted between non-progressors and progressors.

7.5.2 Fas Ligand mRNA Expression by Real Time PCR

The mean expression of Fas ligand for normal nephrectomy tissue was 0.22 (SEM ±0.03). This compares to a mean Fas ligand expression of 0.61 (SEM ±0.15) for all patients with IgA nephropathy, both non-progressors and progressors. This difference was not statistically significant. (Figure 7.7)

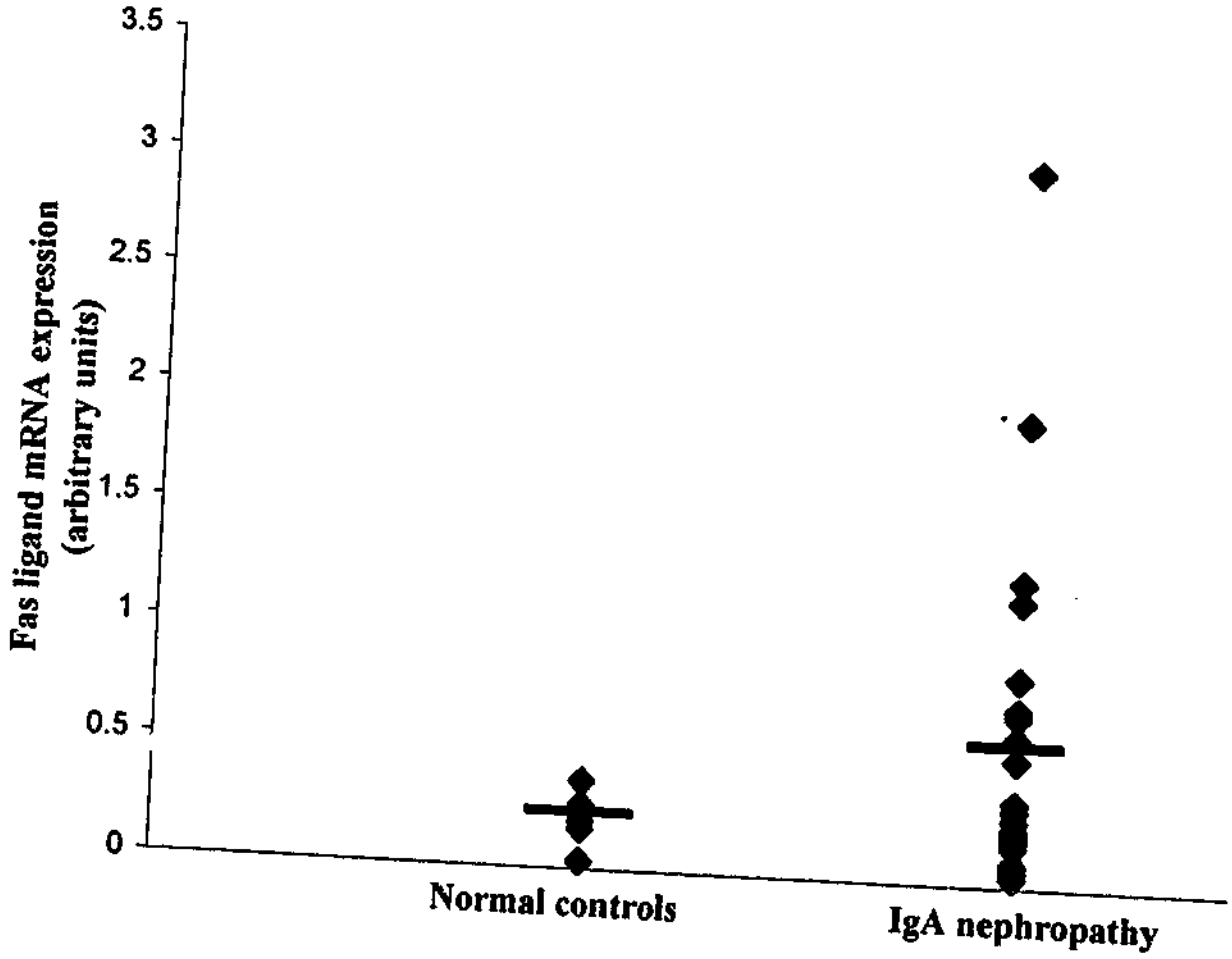

Figure 7.7 Expression of Fas ligand mRNA, normal controls versus IgA nephropathy. Solid bars represent means. No significant difference was noted.

The mean Fas ligand expression for patients with non-progressive disease was 0.46 (SEM ±0.22) compared to a mean of 0.82 (SEM ±0.19) for patients with progressive disease. This represented a statistically significant difference ($p<0.02$). A similar significant difference was noted between normal controls and progressors ($p<0.02$) but no significant difference with found in Fas-ligand expression between normal controls and non-progressors. (Table 7.2 and Figure 7.8)

Patient Group	**Expression of Fas ligand mRNA Mean (±SEM) (arbitrary units)**
Normal (n=7)	0.22 (±0.03)
Non-Progressors (n=13)	0.46 (±0.22)**
Progressors (n=8)	0.82 (±0.19)**

Table 7.2 Mean expression of Fas ligand mRNA for the three patient groups. ** A significant difference was noted between non-progressors and progressors.

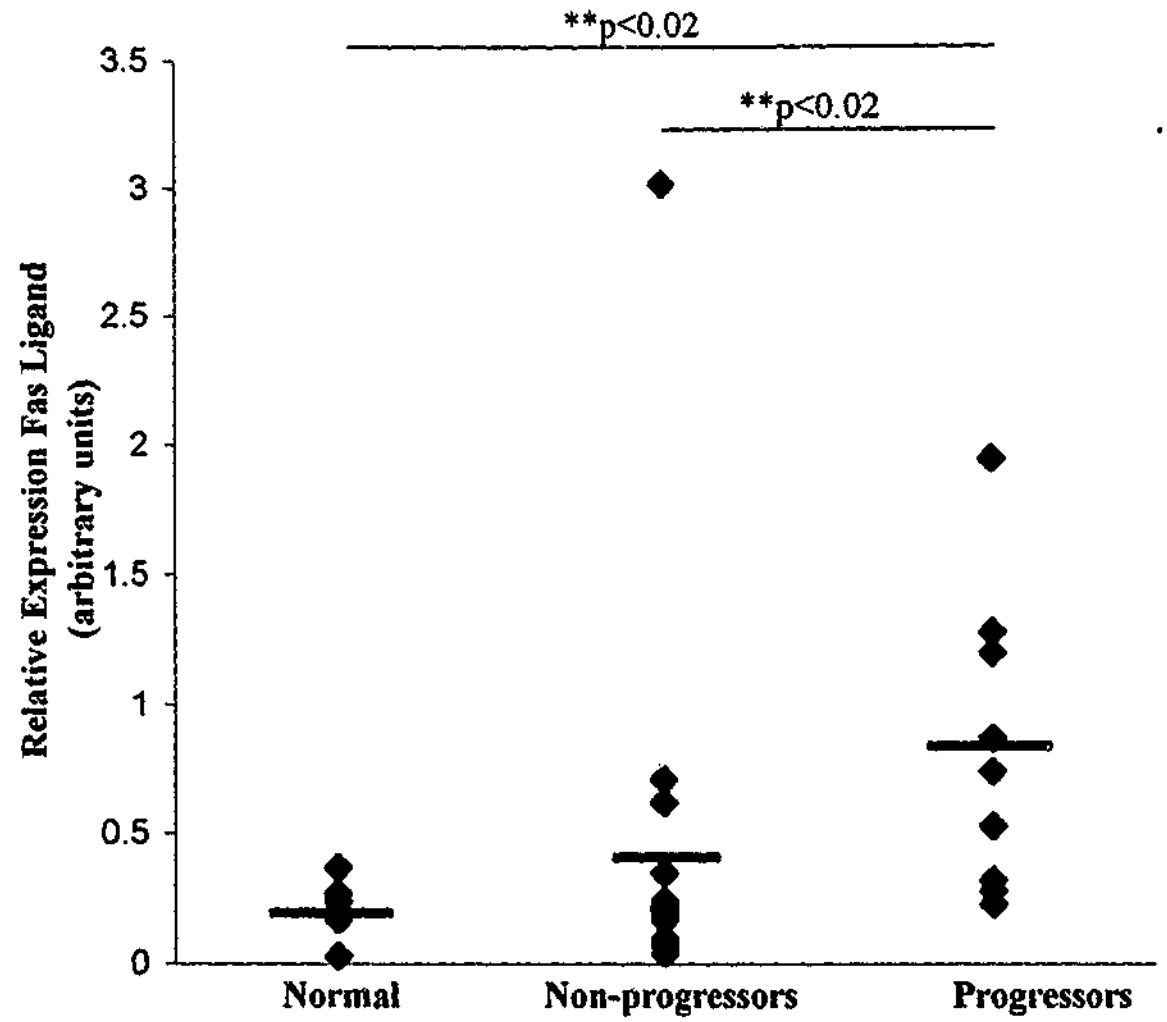

Figure 7.8 Fas ligand mRNA expression, normal versus progressors versus non-progressors. Solid bars represent means. ** A significant difference in Fas ligand expression was noted between normal and progressors as well as between non-progressors and progressors.

7.5.3 Correlations with Fas and Fas Ligand mRNA Expression

The relative expression of the two genes of interest, Fas and Fas ligand were then correlated with other measured variables, both clinical and histological as described in Section 7.4. This was done in order to demonstrate any significant association between the two genes and the other measured variables for the two patient groups.

As expected from the previous analysis in Section 7.5.1 and 7.5.2, the measured expression of Fas and Fas-ligand correlated with disease progression with p values of <0.04 and <0.005 respectively. It must be remembered that for this section of statistical analysis a p value of less than 0.02 was considered significant. The association between Fas and disease progression therefore was not statistically significant ($p<0.04$). Despite this, the expression of both Fas-ligand *and* Fas correlated closely with the other surrogate markers of disease progression already established by the candidate. These include serum creatinine at biopsy, proteinuria at biopsy and sclerosing injury score. (Table 7.3)

The expression of Fas and Fas ligand in the biopsy specimens also correlated with patient age. (Table 7.3) It should be recalled that on univariate analysis patient age at the time of biopsy was significantly associated with disease progression. This may explain this apparent association. It must be noted that the age of patients used as negative controls, i.e. nephrectomy samples was not included in the analysis. This may have had an impact on this correlation.

The most significant association demonstrated was between the expression of Fas and Fas-ligand themselves ($p<0.0001$) (Table 7.3). This is not surprising given the relationship already described between these two genes.

There was no significant correlation between Fas and Fas ligand expression and proliferative injury on biopsy. As well as this there was no association between Fas or Fas ligand expression and Bax or Bcl-2 expression.

Variable	Fas	Fas Ligand
Disease Progression	p <0.04	p <0.005**
Serum Creatinine	p <0.002**	p<0.001**
Proteinuria	p <0.02**	p <0.05
Sclerosing Injury Score	p <0.006**	p <0.002**
Patient Age	p <0.02**	p <0.03
Fas Ligand	p <0.0001**	-

Table 7.3 Significant associations between Fas and Fas ligand mRNA expression and other measured variables. ** Denotes p value < 0.02, the threshold for significance.

7.6 Discussion

In this section of the thesis observations have been made relating to the expression of the mRNA for the cell surface receptor Fas, and its naturally occurring ligand, in extracted mRNA from renal biopsy tissues. The relatively new technique of real-time PCR was utilised with some initial difficulties but eventually a reliable methodology was established.

It has been demonstrated that the expression of Fas-ligand in the extracted mRNA correlated strongly with disease progression in IgA nephropathy as well as with other surrogate markers of disease progression. These included serum creatinine concentration at biopsy, proteinuria and sclerosing injury score. Fas expression did not correlate with disease progression ($p<0.04$). Of note Fas expression *did* correlate significantly with the other markers of disease progression suggesting that a strong association does exist but was not clearly able to be demonstrated with the small patient numbers.

As was demonstrated with immunohistochemistry in Chapter 5, Fas-ligand was largely expressed by the tubular epithelial cells. Unfortunately the expression of Fas was not measured using this technique as a reliable anti-human antibody was not

available at that time. Others however, have been able to document renal tubular expression of Fas *in vitro* and *in vivo* [189, 190]. It is thus likely that the measured Fas and Fas-ligand mRNA in the biopsy extractions originates from the tubular epithelial cells. It is possible that glomerular expression of the two proteins could also contribute to the measured Fas and Fas-ligand in the tissue extractions although the candidate did not observe any Fas-ligand expressed within glomeruli by immunohistochemistry. Others however have been able to demonstrate the expression of both Fas and Fas-ligand in glomeruli in human IgA nephropathy [146]. Again *in-situ* hybridisation for Fas and Fas ligand would potentially clarify this issue.

The association between disease progression and the expression of Fas and Fas-ligand strongly suggests that Fas-induced tubular apoptosis is an important causative factor in the pathogenesis of the tubular atrophy associated with the progressive disease. At the very least the interaction of Fas with its ligand between neighbouring tubular cells increases tubular epithelial cell susceptibility to other apoptosis inducing stimuli. The factors that are known to induce the up-regulation of tubular Fas and Fas-ligand expression such as proteinuria or tubular ischaemia are common in progressive glomerular disease.

Of note, the strongest correlation demonstrated was between the expression of Fas and Fas-ligand themselves. This suggests that their induction share a common causality or that increased expression of one of the proteins induces the expression of the other. As mentioned, Schelling and Cleveland [145] were able to demonstrate *in vitro* evidence of the former, with both interleukin -1 alpha and tumour necrosis factor-alpha inducing increased expression of both Fas and Fas-ligand in cultured epithelial cells.

There was no association noted between the expression of Fas or Fas-ligand and the expression of Bax or Bcl-2. This supports to some extent the observations reported in Chapter 5 and 6 where increased expression of Bcl-2 in patients with progressive disease reflected mostly protein expression in association with interstitial lymphocytes. The up-regulation of Fas-ligand (and assumedly Fas) was demonstrated to reflect increased *tubular* expression of these proteins. The lack of correlation between these suggests that tubular Fas and Fas ligand up-regulation is not directly linked to the

interstitial lymphocytic infiltration. It is possible that the infiltrate is a secondary response to glomerular and/or tubular injury.

It is interesting to note the lack of correlation between Fas and Fas-ligand mRNA expression and Bax mRNA expression. This is despite the fact that the tubular expression of all of these proteins correlated significantly or was at leased closely linked with disease progression. This suggest that mechanisms that induce increased Fas and Fas-ligand tubular expression are not the same as those that induce increased Bax expression despite the fact that all are associated with cellular apoptosis. It is possible that some cytokines or stimuli, increased in disease progression specifically induce tubular Fas and Fas-ligand expression whilst others act upon the Bcl-2 family of proteins to induce apoptosis. Thus it is possible that several independent mechanisms exist for the induction of tubular apoptosis in progressive glomerulonephritis, suggesting that blockade of only one of these mechanisms will not suffice in ameliorating disease progression.

CHAPTER EIGHT

DISCUSSION AND FUTURE DIRECTIONS

8.1 Introduction

There are many limitations associated with the study of human glomerulonephritis, especially one such as IgA nephropathy that is slow to progress and variable in its prognosis. Many of these limitations have been alluded to already by the candidate. Despite this, the importance of human studies must not be underestimated if human disease is to be truly understood. As discussed, animal models of human glomerulonephritis such as IgA nephropathy only approximate the disease process at best.

Therefore the candidate's work, whilst limited to a small group of patients using only remnants of renal biopsy tissue from a single time point in a disease that can take many years to develop is unique in its direct application to the understanding of human glomerular disease. As has been discussed in the literature review, despite the high incidence of IgA nephropathy in the Western World, very little has been published that directly examines the role of apoptosis in the progression of disease.

8.2 Methodology

For the most part the methodologies utilised by the candidate had been established in the laboratory by previous researchers and all that was required was optimisation and minor refinement of the techniques. However, for the application of the relatively new technology of real-time PCR the candidate was required to develop a reliable methodology of his own. The difficulties encountered in establishing this methodology have already been discussed and highlighted a potential source of error in the relative quantitation technique of data analysis, particularly if appropriate validation experiments are not performed. It is unfortunate that the great expense associated with the reagents required for this technique have proved the rate limiting step in further optimisation of the failed methodology.

8.3 IgA Nephropathy Database

The IgA nephropathy database established by the candidate successfully identified a large patient population with primary IgA nephropathy. The clinical and histopathological characteristics of the patient group were collected and follow-up details were obtained where possible. The patient population was demonstrated to closely mirror other reported patient cohorts with perhaps a higher incidence of more progressive disease.

Univariate analysis of the database suggested that most of the measured variables were linked to disease progression. However with the application of the more precise multivariate analysis, only sclerosing injury on biopsy, proteinuria at the time of biopsy and male sex were associated significantly with disease progression. This concurred with most available literature.

The database then proved to be a powerful tool for further analysis of biopsy specimens to be carried out by the candidate. Clear knowledge of an individual patients baseline characteristics as well as disease prognosis allowed for correlation of these parameters with the other measured indices.

8.4 Identification of Apoptotic Cells by TUNEL

The TUNEL technique was successfully utilised to identify apoptotic cells in the renal biopsy specimens. Care had to be taken not to rely upon TUNEL positivity *alone* when identifying apoptotic cells in a test sample. Rather the candidate combined TUNEL positivity with specific morphological features associated with apoptosis to avoid a high incidence of false positivity. This eliminated one source of potential criticism of the TUNEL technique.

Significantly greater numbers of apoptotic cells were identified in the tubulointerstitium of patients with progressive disease compared to patients with non-progressive disease. Glomerular apoptotic cell number did not differ between the two patient groups but total glomerular number was unfortunately relatively low in the biopsy samples. Tubulointerstitial apoptotic cell number was shown to correlate significantly with other surrogate markers of disease progression.

8.5 Inflammatory Infiltrates Associated with IgA Nephropathy

Immunohistochemistry was utilised to identify the nature of the glomerular and interstitial infiltrates in the biopsy specimens examined. It was observed that glomeruli contained relatively few inflammatory cells, mostly macrophage or polymorph in origin. No specific differences were noted between the two patient groups. Infiltrating tubulointerstitial leucocytes were noted to be predominantly T-cell in origin with a lesser contribution from B-cells and macrophages. Greater numbers of B-cells were noted in patients with progressive disease, but T-cell and macrophage number did not differ significantly between the two groups.

Observations made regarding the expression of α-SMA in the two patient groups suggested increased expression of this isoform of actin associated with tubulointerstitial fibrosis in patients with progressive disease.

8.6 Expression of Apoptotic Proteins by Immunohistochemistry

Immunohistochemistry was further utilised to explore the expression of some of the regulatory proteins associated with apoptosis in the biopsy specimens. In particular biopsies were examined for the expression of Bcl-2, Bax, Bcl-X_L and Fas-ligand.

It was observed that Bcl-2 was expressed solely in association with infiltrating leucocytes, Bax was expressed by tubular epithelial cells as well as by infiltrating leucocytes and Bcl-X_L and Fas-ligand were expressed only by tubular epithelial cells. The degree of tubulointerstitial Bcl-2 expression correlated with disease progression as well as with other surrogate markers of disease progression such as proteinuria. As it had been already established that the intensity of the tubulointerstitial infiltrate correlated with disease progression, it was not surprising that this was the case. The expression of the other proteins examined did not correlate with disease progression. This may reflect the crude scoring system that was utilised by the candidate to measure their expression rather than an absence of a true difference between the two patient groups.

Alternatively as discussed, real differences in mRNA gene expression between the two patient groups may not necessarily give rise to detectable differences in protein levels due to inhibition of mRNA translation or protein cleavage.

8.7 Quantification of Apoptotic Proteins by Competitive and Real-time PCR

For more accurate quantification of the expression of apoptotic regulatory proteins in the biopsy specimens, PCR analysis of mRNA extractions from biopsy tissues was undertaken, measuring the expression of Bax, Bcl-2, Fas and Fas-ligand.

The expression of Bax and Bcl-2 both correlated with disease progression on analysis. This concurred with observations made on immunohistochemistry and it was suggested by the candidate that this relationship related to increased interstitial leucocyte expression of the two proteins. The strong correlation noted between the expression of Bax and Bcl-2 themselves was thought to relate to the formation of heterodimers between these two molecules to exert their pro/anti-apoptotic affect.

Fas-ligand and to a lessor extent Fas expression was also noted to correlate with disease progression as well as with the other surrogate markers for disease progression. As noted by immunohistochemical analysis, Fas-ligand expression was limited to tubular epithelial cells and it was suggested by the candidate that this increased Fas-ligand and possibly Fas expression on tubular epithelial cells in patients with progressive disease was a potent inducer of tubular apoptosis and atrophy. The relationship observed between the degree of proteinuria and the expression of these pro-apoptotic proteins suggested one possible mechanism for this up-regulation in pro-apoptotic signals.

8.8 Future Directions

The candidate has clearly demonstrated the role of apoptosis in the development of tubulointerstitial injury in progressive IgA nephropathy. The data presented suggests that this is mediated by increased tubular expression of Fas and Fas-ligand, possibly the result of the increased tubular protein traffic observed in progressive disease. The nature of the tubulointerstitial infiltrate associated with non-progressive and progressive

disease has also been defined. The data suggests a role for increased Bcl-2 expression by leucocytes to inhibit apoptosis of the inflammatory infiltrate, perhaps required for cell survival in the ischaemic and fibrotic milieu of the tubulointerstitium in progressive disease.

Unfortunately with regards to glomerular injury, apart from some observations regarding the inflammatory infiltrate associated with the IgA nephropathy, the candidate has been unable to draw firm conclusions from the data regarding the other aspects of apoptosis examined. This is possibly the result of the tissue samples utilised by the candidate which often contained very few glomeruli. Assessment of further tissue samples as they become available, particularly those containing larger numbers of glomeruli, may allow expansion of the data and possibly demonstrate more significant results. The source of the biopsy tissue will however always make this difficult.

It would have been ideal to perform the various analyses on the *same* group of patients for each section of thesis. Again this was not possible due to the source of renal tissue. However, with further collection of biopsy samples, particularly samples for mRNA extraction this may be possible on a small patient population.

Immunohistochemical analysis of the biopsy specimens for the expression of Fas was not performed due to the unavailability of a reliable antibody, despite testing a number of commercially-made products. If a reliable antibody could be identified, its application to the tissue samples would serve to demonstrate the site of Fas expression, hopefully supporting the candidate's theory on tubular-cell Fas expression in progressive disease.

As has been discussed, some discrepancies have arisen between the results of immunohistochemical analysis of protein expression and mRNA analysis of gene expression, particularly with regard to Bax and Bcl-2. This discrepancy it was suggested may have arisen as a result of the methods of analysis utilised or by the "dilution affect" of whole tissue RNA extraction. The application of *in-situ* hybridisation techniques to the tissue samples may overcome this difficulty and more accurately determine the *source* of increased mRNA expression within the samples.

The database has proved a very useful tool for use in this type of tissue analysis and it is the candidate's intention to maintain the database for as long as practical to

allow further analysis of this patient population by the candidate himself or other members of the laboratory.

To further explore the affect of specific therapies on apoptotic cell number and on the expression of the regulatory proteins examined, it may be useful to collect further information in the database. For example, noting the type of anti-hypertensive therapy used by patients. This would allow for assessment of the benefits of say ACE inhibitors compared to other anti-hypertensive agents in preventing tubular apoptosis, as has been described in animal models of disease. The benefits of steroid or cytotoxic therapy could be similarly examined.

Finally, the study techniques utilised by the candidate could be applied to other forms of glomerulonephritis (GN) as well as forms of non-glomerular renal disease such as transplant rejection. This would allow investigation of the role of apoptosis in the pathogenesis of these disease entities. Of particular interest would be isolating non-proliferative but proteinuric renal disease such as minimal change GN, and exploring the profile of apoptotic proteins expressed. As glomerular proliferation/apoptosis is not a feature of these GNs, the apoptotic proteins expressed are more likely to reflect tubulointerstitial up-regulation alone. This would test the candidate's hypothesis that tubular epithelial cell apoptosis is induced by the increased expression of Fas and Fas-ligand in response to increased protein traffic. Similarly examination of other forms of proliferative GN such as post-infectious GN, in which sustained heavy proteinuria is rare, would allow for assessment of apoptotic protein profiles in the absence of ongoing significant proteinuria. Again the application of *in-situ* hybridisation techniques to these disease entities would further clarify the site of expression of the apoptotic regulatory proteins.

CHAPTER NINE

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