Angiogenesis in Human Parathyroid Disease and Chronic Allograft Nephropathy

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Contents

Preface

I confirm that this thesis has been composed by myself.

I acknowledge that the work reported was done by myself at the Tissue Injury and Repair Group laboratories within the Department of Surgical and Clinical Sciences (Surgery) in Edinburgh, where I have worked for the last two years. Exceptions to this are;

- VEGF in situ hybridisation which was carried out in collaboration with Helen Wilson at the MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, Royal Infirmary of Edinburgh

- Parathyroid CD31 staining carried out by Lorna Marson of the Tissue Injury and Repair Group

- Parathyroid VEGF staining carried out by Lisa Sanders of the Tissue Injury and Repair Group

- Protocol biopsy CD31 staining carried out in collaboration with Sue Fuggle at the Oxford Transplant Centre

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Abstracts

Angiogenesis in parathyroid disease and association with VEGF

Angiogenesis has been demonstrated in normal parathyroid tissue in vitro and must occur in vivo to allow for successful transplantation. Here we present data that angiogenesis also occurs in parathyroid disease states and that VEGF (a known endothelial cell mitogen) expression is not evident in these tissues.

Formalin fixed, paraffin embedded sections of human parathyroid tissue were stained with an antibody to CD31 to visualise endothelial cells and separate sections stained with antibody to VEGF using standard immunohistochemistry techniques. Microvessel counts were performed by two (blinded) investigators. Western blot analysis of protein lysate of parathyroid tissue was also performed using antibody to VEGF.

123 patients were studied (44 normal, 27 hyperplasia and 52 adenoma). Microvessel counts showed a statistically significant ($p < 0.05$ compared with normal) and step-wise increase in microvessel count between the different types of tissue.120 parathyroid sections stained with antibody to VEGF with positive control of normal kidney showed non-specific staining in all tissue types, a finding which was repeated with Western blot analysis of protein derived from 1 normal, 2 adenomas and 2 hyperplasia samples.

Here we have demonstrated evidence of angiogenesis that occurs in human parathyroid tissue when diseased. Our data have not shown a significant expression of VEGF in these samples. This finding implies that either VEGF does not mediate angiogenesis in these tissues or that it does so transiently. Alternatively VEGF may act in concentrations too small to be detected with these techniques. Gaining a better understanding of the progression of angiogenesis in parathyroid disease may lead to the development of novel therapeutic strategies.

Changes in Vascularity Associated with Chronic Allograft Nephropathy

This study aims to compare vascularity of kidneys with CAN versus normal. In addition, validation of renal biopsy samples in the assessment of angiogenesis has been performed.

Tissue from transplant nephrectomies performed because of CAN (n=29), antecedent biopsies from these grafts (n=18) and normal kidneys (n=61) were studied. Protocol renal transplant biopsies were also studied. Tissue sections were immunostained with the endothelial cell antibody to CD31. Changes in overall vascular patterns were noted and microvessel counts performed. Sections were dual-stained with antibody to CD31 and the proliferation marker, MIB-1, to determine the presence of proliferating endothelial cells. Microvessel counts were performed on paired samples of core biopsies and cross-section of normal kidney and were compared in early protocol biopsy specimens from grafts which develop CAN (n=10) with those where stable graft function continued (n=20). Macrophage infiltration was studied using immunostaining to CD68.

CD31 staining from CAN nephrectomies exhibited diminished cortical and medullary staining and was accompanied by a significant increase in proliferation index when compared with normal. There was significant correlation in microvessel counts in core biopsies and the corresponding kidney cross-section. Early biopsies from grafts which developed CAN show significantly higher microvessel counts compared with the corresponding nephrectomy. The number of proliferating ECs was significantly increased in this biopsy group compared with normal kidney but was not significantly different compared with the CAN nephrectomy group. Early protocol biopsies from grafts which developed CAN showed significantly higher microvessel counts compared with those with stable graft function. A significant increase in macrophage infiltration was seen in CAN nephrectomies compared with normal kidney.

This study demonstrates reduced density of CD31–positive microvessels in CAN compared with normal. In addition, the investigation of changes in the microvasculature in CAN by study of core biopsies has been validated and confirms preservation of normal vasculature in early CAN. Together with evidence of proliferating endothelial cells these findings support a hypothesis that, early in the development of CAN, angiogenesis is stimulated, but despite this attempt at tissue repair, progressive microvascular loss occurs. The presence of significant numbers of macrophages may be of aetiological importance or reflect changes relating to cessation of immunosuppression.

Chapter 1; Introduction

This thesis examines changes in vascularity in two diseases affecting patients with end stage renal failure: parathyroid disease and chronic allograft nephropathy. In clinical practice, chronic renal failure (CRF) links these two conditions. CRF is associated with secondary hyperparathyroidism due to failure of kidneys to metabolise vitamin D effectively. One therapeutic option for end stage renal failure is transplantation and a common cause of loss of kidney transplants after one year is chronic allograft nephropathy (CAN).

The work undertaken in this study will present data for each condition separately. Common background and methodology appear in the appropriate sections.

Parathyroid function

Parathyroid glands in mammals play a central role in the regulation of calcium homeostasis through secretion of parathyroid hormone. This 84 amino acid peptide has a half life of a few minutes and acts on cell membrane receptors to increase cellular cAMP. It acts on bone, kidney and gut (Cohn et al 1986).

Table 1.1: End organ effects of PTH

Tissue	Action
Bone	Increases turnover of bone and stimulates calcium release
Kidney	Increases production of 1,25 dihydroxy-vitamin D3
Gut	Increases calcium absorption from small intestine

Secretion of parathyroid hormone increases in response to low levels of serum calcium such that calcium resorption from bone occurs, renal losses of calcium reduce and absorption from the gut increases until normal levels of calcium are restored (Cohn et al 1986, Brown et al 1995, Slatopolsky et al 1999). In the majority of the human population there are four parathyroid glands situated in the neck, although ~4% of people will have more than four glands and up to 25% of patients may have supernumerary and/or aberrant glands.

The differential diagnoses in hypercalcaemia are:

- primary hyperparathyroidism
- renal failure and secondary or tertiary hyperparathyroidism
- malignancy
- granulomatous disease e.g. sarcoid, TB
- drugs e.g. thiazide diurectics, vitamin D toxicity, lithium
- endocrine causes e.g. thyrotoxicosis, adrenal crisis
- immobilisation
- aluminium intoxication

Disease states

Hyperparathyroidism is a condition in which increased levels of parathyroid hormone are found in the blood. Three main types are recognized: primary, secondary and tertiary. In the primary form increased parathyroid hormone secretion occurs as a result of abnormality in one or more of the parathyroid glands, usually owing to the presence of adenoma or rarely carcinoma. In this form the fundamental biochemical finding is persistent hypercalcaemia. The secondary form is associated with abnormalities of parathyroid function induced by a sustained hypocalcaemic stimulus, often resulting from chronic renal failure or malabsorption states. In secondary hyperparathyroidism, renal abnormality is associated with additional soft tissue and skeletal changes, and the entire complex is termed renal osteodystrophy (Akizawa et al 1999, Beckerman et al 1999). Tertiary hyperparathyroidism is found in patients with chronic renal failure or malabsorption and secondary hyperparathyroidism of long duration who develop relatively autonomous parathyroid function and hypercalcaemia (Fukagawa 1999). This situation also occurs following renal transplantation when the stimulus to PTH secretion has been removed but parathyroid glands function autonomously. The clinical features of primary and secondary hyperparathyroidism differ in some respects (Table 1.2).

Table 1.2. Primary versus secondary hyperparathyroidism.

Findings	**Primary hyperparathyroidism**	**Secondary hyperparathyroidism**
Brown tumours	Common	Less common
Osteosclerosis	Rare	Common
Chondrocalcinosis	Not infrequent	Rare
Periostitis	Rare	Not infrequent

Hyperparathyroidism leads to considerable bone erosion involving subperiosteal, intracortical, endosteal, trabecular, subchondral and subligamentous foci. In renal osteodystrophy, additional features are noted, including osteomalacia, osteoporosis, and soft tissue and vascular calcification. Haemodialysis and renal transplantation may cause these findings to become exaggerated or arrested.

Bone tissue in hyperparathyroidism demonstrates osteitis fibrosa cystica, with replacement of marrow elements by highly vascular fibrous tissue, as well as osteoporosis and osteomalacia. Localized cysts or brown tumours may also be seen.

Bone resorption in the hands can be identified in the early stages of the disease by high quality radiography. Subperiosteal resorption of cortical bone is virtually diagnostic of hyperparathyroid bone disease. Brown tumours, representing localized accumulations of fibrous tissue and giant cells, can replace bone and even may produce osseous expansion.

Brown tumours appear as single or multiple well-defined lesions of the axial or appendicular skeleton. Common sites of involvement are the facial bones, pelvis, ribs and femora.

Bone sclerosis, marked by diffuse increase in bone density, may be apparent in the metaphyseal regions of the long bones, the skull or the vertebral endplates. In addition, deposition of bone in the subchondral areas of the vertebral bodies leads to the appearance of radiodense bands across the superior and inferior margins (rugger jersey spine).

Primary hyperparathyroidism is frequently associated with chondrocalcinosis. Other rheumatologic manifestations include capsular and ligamentous laxity, as well as rupture, contributing to joint instability, traumatic synovitis, and cartilaginous and osseous destruction. Monosodium urate crystal deposition and clinical gout have also been described in patients with hyperparathyroidism.

Treatment of Parathyroid Disease

Primary hyperparathyroidism is diagnosed when a patient presents with an elevated serum calcium level in conjunction with elevated levels of parathyroid hormone. In these cases an adenoma of one of the parathyroid glands is seen although occasionally more than one gland may be affected. In the patient fit to undergo surgery the treatment of choice is excision of the affected gland. Because three glands are left in the patient calcium homeostasis is restored in the majority of cases.

Secondary hyperparathyroidism occurs when there is appropriate increase in the secretion of parathyroid hormone in response to a persistently low level of serum calcium as can occur in chronic renal failure where it is related to the inability of failing kidneys to convert vitamin D to its active form. All four parathyroid glands are affected with histological features of hyperplasia. Overt osteodystrophy will develop in 10% to 25% of patients receiving long-term dialysis treatment (Mozes et al 1980). Treatment in this instance will sometimes require excision of the affected parathyroid tissue and this can be done using one of three methods:

- total parathyroidectomy with lifelong calcium and/or vitamin D supplementaion,

- total parathyroidectomy with autotransplantation of a small amount of parathyroid tissue into forearm muscle (Wells et al 1975)

- subtotal parathyroidectomy where the majority of parathyroid tissue is excised but a portion of one vascular gland is left within the neck.

In each instance the aim of treatment is restoration of normal calcium homeostasis and parathyroid function with the hope that further surgery will not be required to treat recurrent disease. Each method has advantages and disadvantages. With total parathyroidectomy and autotransplantation over 90% of grafts function successfully (Takagi et al 1983) and with appropriate PTH release in response to changes in

serum calcium concentrations (Schneider et al 1977, Brown et al 1979) but in those that do not there is no remaining functioning parathyroid tissue with the patient requiring lifelong calcium supplementation. This method avoids the need for a second and more difficult neck dissection close to vital structures, such as the recurrent laryngeal nerve, should disease recur. Excision of parathyroid implants from forearm muscle is safer and can often be performed under local anaesthesia. In the long term the majority of these implants will require excision due to disease recurrence. By performing a subtotal parathyroidectomy the uncertainty over graft function is removed and restoration of calcium homeostasis is achieved. However there is a risk that insufficient functioning parathyroid tissue remains either due to excision of too much of the gland or vascular compromise to the remaining gland. Should disease recur, further challenging neck surgery is required with associated morbidity in some cases.

Further research is required to identify the precise mechanisms by which parathyroid glands become diseased. Surgical debate will continue as to which operative method is of greatest benefit and central to this discussion are the criteria used for evaluating success (Saxe 1984). Medical control of secondary hyperparathyroidism, obviating the need for surgery, may be a realistic therapeutic target for most patients but current therapies do not yet achieve this (Goodman 2004a).

Current therapeutic strategies rely largely on the use of vitamin D sterols to diminish excess PTH synthesis and to lower serum or plasma PTH levels. However their use is often confounded by increases in serum calcium or phosphate concentrations which can aggravate soft tissue and vascular calcification (Goodman 2004a). Thus the development of new, more physiologically relevant therapies which may lead to the successful management of these conditions is needed (Moe et al 2003). One area of interest is the control of neovascularisation in the diseased gland

The process of angiogenesis is the focus of interest in this thesis.

Angiogenesis

In this section, the process of angiogenesis is outlined and mechanisms by which it is mediated considered.

Specific reference to angiogenesis in parathyroid disease is made in the discussion of chapter 3 and a detailed review of a known inducer of angiogenesis, vascular endothelial growth factor, is presented.

The role of angiogenesis during the development of chronic allograft nephropathy is discussed in a later section in this chapter which details proposed pathogenesis of that condition.

Physiological angiogenesis

Angiogenesis is an important physiological process that first occurs in the developing embryo where the cardiovascular system is the first organ system to develop and reach functional maturity. It is defined as the growth of new blood vessels from the endothelium of existing vessels. The term vasculogenesis defines the process of new blood vessel growth from endothelial cell precursors. Both are evident during embryonic development, but in the normal adult male angiogenesis is quiescent. 'Physiological' angiogenesis occurs in the adult during the process of wound healing and as part of the female reproductive cycle (ovulation, menstruation and placental development) where new blood vessel growth is essential (Wulff et al 2000). 'Pathological' angiogenesis has been implicated in a number of proliferative disorders including benign conditions such as retinopathies (Gole et al 1990), rheumatoid arthritis, Crohn's disease, psoriasis (Colville-Nash et al 1992) as well as in malignancy.

Angiogenesis is a multistep process involving alterations in endothelial cells and extracellular matrix remodelling. Figure 1 shows a representation of the steps involved during tumour induced angiogenesis.

There is local degradation of basement membrane caused by the release of proteases in the extracellular matrix and endothelial cells around the site of disruption change shape and invade stroma. These endothelial cells are stimulated to proliferate at the leading edge of the migrating column, adhering to each other tightly to form tubes which coalesce to form loops. At this point blood starts to circulate in newly formed vessels. Endothelial cell proliferation therefore forms an important part of the process of angiogenesis and investigation of the presence of proliferating endothelial cells plays an important part in the studies presented here.

Figure 1: Diagramatic representation of angiogenesis

Tumour angiogenesis

The majority of published research to date involves the study of angiogenesis during tumour development as this provides an exciting, potential novel therapeutic target in the treatment of cancer. Angiogenesis has been shown to be central to both the growth and metastasis of solid tumours. This complex angiogenic process involves multiple paracrine and autocrine interactions of stromal and tumour components (Casey et al 1995, Weidner et al 1992) and the mechanisms of control involve both stimulatory and inhibitory factors (Hanahan et al 1996). During tumour development, new vessel formation occurs as an early event and is a discrete component of the tumour phenotype rather than resulting from tumour hypoxia as the growing mass outgrows its blood supply (Hanahan et al 1996).

Post-mortem examination of individuals who died of trauma revealed high numbers of in situ carcinoma. The most striking finding was that virtually all individuals aged 50 to 70 had in situ carcinomas in their thyroid glands despite the fact that only 0.1% of this age group are diagnosed with thyroid cancer during this period of their life (Folkman 2004). The suggestion is that many of us carry in situ tumours but that these are mostly dormant and need additional signals to grow and become potentially life-threatening cancers. The two-phase theory is thus:

Phase 1: Acquisition of mutations leads to transformation of normal cells into cancer cells.

Phase 2: This involves a switch to the angiogenic phenotype, due to constant recruitment of new blood vessels, which converts non-lethal in situ tumours into an expanding mass of tumour cells.

It is anticipated that advances made in the field of angiogenesis in tumour biology, specifically anti-angiogenic strategies that result in the regression of blood vessels accompanied by tumour cell apoptosis, will be applicable to other settings where modulation of this process will confer benefit to the patient.

Regulation of angiogenesis

Recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes requiring the sequential activation of a series of receptors by numerous ligands. Angiogenesis requires activation of quiescent endothelial cells by growth factors, degradation of the basement membrane and dissociation from the supporting vascular smooth muscle. These cells must proliferate, survive, migrate and then differentiate into lumen-bearing structures that may also be lined with vascular smooth muscle cells. Inhibitors of angiogenesis may block or alter any of these discrete processes and it is the development of therapeutic agents that act in this way that provides the focus of clinical trials with anti-angiogenic agents.

Vascular endothelial cell growth factor (VEGF) signalling often represents a crucial rate-limiting step in physiological angiogenesis and is important in pathological angiogenesis such as that which occurs during tumour progression. Development of anti-VEGF monoclonal antibodies that have been used in phase 2 clinical trials have provided evidence of clinical efficacy in the treatment of human tumours (Ferrara 2002, Ferrara 2004b).

An angiogenic response has been demonstrated by tissue derived from human parathyroid samples with a histological diagnosis of hyperplasia and adenoma. Using an in vitro model of angiogenesis, normal canine and human parathyroid tissue have been shown to stimulate new vessel growth in vitro. Results from the same model of angiogenesis have shown that, following explant, there is a twelve fold increase in the expression of vascular endothelial growth factor (VEGF) mRNA as shown by quantitative reverse transcription polymerase chain reaction (rtPCR) analysis in human parathyroid cells and furthermore that the angiogenesis stimulated by this tissue is completely obliterated by coculture with FLT-1 soluble fusion protein (Carter et al 2000). This work strongly implicates VEGF as being an important mediator of parathyroid-induced angiogenesis in this model and it has further been shown that angiopoietin-2 modulates this response (Carter et al 2001).

Vascular endothelial growth factor (VEGF)

One of the most important endothelial growth and survival factors is vascular endothelial growth factor (VEGF) (Ferrara 1999, Ferrara 2004a, Ferrara 2005). VEGF induces angiogenesis and endothelial cell proliferation and plays an important role in regulating vasculogenesis. VEGF is a heparin-binding glycoprotein that is secreted as a homodimer of 45 kDa (Houck et al 1992). Most types of cells, but usually not endothelial cells themselves, secrete VEGF. VEGF-A increases vascular permeability and it was known as vascular permeability factor (Dvorak et al. 1999). In addition, VEGF causes vasodilatation, partly through stimulation of nitric oxide synthase in endothelial cells (Yang et al 1996) and can also stimulate cell migration and inhibit apoptosis (Alon et al (1995).

There are several splice variants of VEGF-A. The major ones include: 121, 165, 189 and 206 amino acids (aa), each one comprising a specific exon addition. $VEGF_{165}$ is the most predominant protein, but transcripts of $VEGF_{121}$ may be more abundant (Relf et al 1997). The 165, 189 and 206 aa splice variants have heparin-binding domains, which help anchor them in extracellular matrix and are involved in binding to heparin sulfate and presentation to VEGF receptors. This is a key factor for VEGF potency (*i.e.*, the heparin-binding forms are more active). Several other members of the VEGF family have been cloned including VEGF-B, -C, and –D (Eriksson et al 1999, Carmeliet et al 1999). Placenta growth factor (PlGF) is also closely related to VEGF-A. VEGF-A, -B, -C, -D, and PlGF are all distantly related to platelet-derived growth factors-A and -B. Less is known about the function and regulation of VEGF-B, -C, and -D, but they do not seem to be regulated by the major pathways that regulate VEGF-A.

VEGF-A transcription is potentiated in response to hypoxia and by activated oncogenes. The transcription factors, hypoxia inducible factor-1α (HIF-1α) and -2α, are degraded by proteosomes in normoxia and stabilized in hypoxia (Wegner et al 1997, Kallio et al 1999). This pathway is dependent on the Von Hippel-Lindau gene product (Maxwell et al 1999). HIF-α and HIF-2 α heterodimerize with the aryl

hydrocarbon nuclear translocator in the nucleus and bind the VEGF promoter/enhancer. This is a key pathway expressed in most types of cells. Hypoxia inducibility, in particular, characterizes VEGF-A compared with other members of the VEGF family and other angiogenic factors. VEGF transcription in normoxia is activated by many oncogenes and these pathways together account for a marked upregulation of VEGF-A in tumours compared to normal tissues and are often of prognostic importance (Gaspirini 1997).

There are three receptors in the VEGF receptor family (Shibuya et al 1999, Kaipainen et al 1997). They have the common properties of multiple IgG-like extracellular domains and tyrosine kinase activity. The enzyme domains of VEGF receptor 1 (VEGF R1, also known as Flt-1), VEGF R2 (also known as KDR or Flk-1), and VEGF R3 (also known as Flt-4) are divided by an inserted sequence. Endothelial cells also express additional VEGF receptors, Neuropilin-1 and Neuropilin-2. VEGF-A binds to Flt-1, KDR and to Neuropilin-1 and Neuropilin-2. PlGF and VEGF-B bind Flt-1 and Neuropilin-1, VEGF-C and -D bind Flt-4 and KDR (Neufeld et al 1999, Makinen et al 1999, Migdal et al 1998).

The VEGF-C/Flt-4 pathway is important for lymphatic proliferation (Jeltsch et al 1997). Flt-4 is specifically expressed on lymphatic endothelium. A soluble form of Flt-1 can be detected in peripheral blood and is a high affinity ligand for VEGF (Aiello et al 1995). Soluble Flt-1 can be used to antagonize VEGF function. Flt-1 and KDR are upregulated on tumour and proliferating endothelium, partly by hypoxia and also in response to VEGF-A itself. Flt-1 is of higher affinity than KDR and mediates motility and vascular permeability. KDR is necessary for proliferation.

VEGF can be detected in both plasma and serum samples of patients, with much higher levels in serum (Banks et al 1998). Platelets release VEGF upon aggregation and may be a major source of VEGF delivery to tumours (Pinedo et al 1998). Several studies have shown that the association of high serum levels of VEGF with poor

prognosis in cancer patients may be correlated with an elevated platelet count (O'Byrne et al 1999). Many tumours release cytokines that can stimulate the production of megakaryocytes in the marrow and elevate the platelet count. This can result in an indirect increase of VEGF delivery to tumours (Salgado et al 1999).

VEGF is implicated in several other pathological conditions associated with enhanced angiogenesis. For example, VEGF plays a role in both psoriasis and rheumatoid arthritis (Koch et al 1994). Diabetic retinopathy is associated with high intraocular levels of VEGF. Inhibition of VEGF function may result in infertility by blockade of corpus luteum function (Ferrara et al 1998). Interference with VEGF function has therefore become of major interest in the development of drugs to block angiogenesis. Approaches include antagonists of VEGF or its receptors, selective tyrosine kinase inhibitors, targeting of drugs and toxins to VEGF receptors, and gene therapy regulated by the same hypoxia pathway that controls VEGF production. Targeting the VEGF signalling pathway may be of major therapeutic importance for many diseases.

Therapeutic applications

There is intense research to develop new anti-cancer drugs which target angiogenesis. Such agents can target either tumour derived pro-angiogenic factors such as VEGF or boost our natural anti-angiogenic defences with the aim to achieve a balance that prevents further blood vessel growth or causes regression of blood supply.

Anti-angiogenic strategies might be expected to increase tumour hypoxia and thus decrease the efficacy of chemotherapy or radiotherapy. It has been shown however that anti-angiogenic therapy actually increases the efficacy of these conventional therapies (Hess et al 2001). VEGF has been shown to have direct effects on at least some tumour cells, acting as a survival factor and protecting them from apoptosis-inducing therapies (Harmey et al 2002). VEGF is also induced by many chemotherapy/radiotherapy regimens and may contribute to tumour resistance. By reversing the increase in vascular permeability induced by VEGF, interstitial fluid pressure decreases and partial pressure of oxygen increases rendering the tumour susceptible to other therapies.

Anti-VEGF monoclonal antibody therapies have demonstrated significant blockade of tumour angiogenesis in rhabdomyosarcoma (Ferrara 2002) and adenovirus mediated soluble flt-1 VEGF receptor has significantly suppressed tumour growth and microvessel density in human pancreatic cells (Hoshida et al 2001).

Clinical trials of angiogenesis inhibitors to treat cancer require different guidelines for their optimum use in animal models and humans (Folkman 2003). A double-blind placebo controlled phase II has shown a significant increase in time to tumour progression in renal cell carcinoma patients that were treated with an anti-VEGF humanized monoclonal antibody (Feldman et al 2002). Such beneficial effects are not without side effect. TNP-470 is an angiogenesis inhibitor which has been shown to slow tumour growth in patients with metastatic cancer in clinical trials. However, at higher doses necessary for tumour regression, many patients experienced neurotoxicity (Satchi-Fainaro et al 2004). Polymer conjugation prevented TNP-470

from crossing the blood brain barrier and decreased its accumulation in normal organs and this should be the aim of these therapies.

The majority of work presented in this thesis does not involve the study of cancer. However, it is necessary to understand tumour angiogenesis and therapies directed at angiogenesis if these advances are to be translated into other areas of biology.

Chronic Allograft Nephropathy

1) overview/epidemiology
2) clinical findings/diagnosis
3) histopathological features
4) risk factors/prognostic indicators
5) proposed pathogenesis of CAN development
6) treatment options

Overview

Chronic allograft nephropathy (CAN) is one of the most common causes of renal transplant failure in the first post-transplant decade and the pathogenesis of this condition remains unclear. CAN accounts for up to 40% of grafts which develop progressive dysfunction and failure after the initial post-transplant months despite appropriate treatment of acute rejection episodes that occur during this time (Paul 1999). Improvements in modern immunosuppressive therapy has resulted in dramatic improvement in graft survival rates in the short term (the one-year cadaveric allograft survival is around 90%) (Ponticelli 2002) but there has only been modest improvement in the long-term graft survival rates. This provides a focus for the current research.

The term chronic allograft nephropathy has evolved to reflect the multifactorial nature of the pathogenesis of this condition in which immune and non-immune factors are known to be implicated and the diagnosis is secured by a combination of clinical and histological criteria.

Once diagnosed, the condition follows a variable clinical course that may result in graft loss and a return to renal replacement therapy. At present, although recent studies have suggested that optimisation of immunosuppressive regimens will maximise graft function, in particular the use of calcineurin inhibitor sparing agents thus limiting nephrotoxicity, there remains no definitive treatment for established

CAN (Gonzalez-Molina et al 2002, Weir 2001, Weir et al 2001, Pilmore et al 2002, Pretagostini et al 2001).

Mechanistic and molecular understanding of the pathogenesis of CAN is needed if novel therapeutic strategies are to be developed to prevent or delay its development. Identification of risk factors in both donor and recipient may allow optimisation of the graft and further understanding of the relative importance of various insults sustained by the graft will lead to the development of new therapeutic strategies for their prevention.

Clinical diagnosis of CAN

The European Renal Association – European Dialysis Transplantation Association (ERA-EDTA) created the European Best Practice Guidelines in 1998. More recently published guidelines (Nephrology Dialysis Transplantation 2002) have focused on the long-term management of the transplant recipient. These guidelines address topics such as the organization of follow-up after the first transplant year, the differential diagnosis of chronic graft dysfunction, the control of alloimmune and non-immune factors and the management of de novo renal disease following transplantation. A brief summary of these guidelines follows.

Organization of follow-up

Guideline A: All renal transplant recipients should undergo regular laboratory check-ups and regular out-patients visits.

Guideline B: All renal transplant recipients should be seen at least once a year in the transplant centre where the transplant was performed or referred to a closer transplant centre.

Such follow-up will allow for the specialist treatment of delayed complications after transplantation and for the prompt diagnosis of CAN.

Chronic graft dysfunction

This term represents a clinical diagnosis and is variably defined in the literature. Consistent to many definitions of chronic graft dysfunction is that there is a progressive decline in graft function after the first three post-transplant months as shown by a rise in serum creatinine levels, the presence of proteinuria or a decline in graft glomerular filtration rate (GFR). Other clinical manifestations, such as the presence of systemic hypertension, may coexist but are less specific to graft dysfunction owing to its high prevalence (Raine 1995).

Chronic graft dysfunction differs from delayed graft function which occurs at an earlier stage. Delayed graft function is defined as the requirement of dialysis in a specified post-operative period, usually the first week. Other studies also include urine output and a decrease in serum creatinine as criteria to distinguish delayed graft function from early function (Shoskes et al 1996). Although the two diagnoses are distinct there is evidence to suggest that early graft damage and discrete events secondary to vascular rejection and delayed graft function have an effect on the development of CAN (Nankivell et al 2001). The pathogenesis and risk factors for the development of CAN will be discussed in greater detail below.

The EBPG guidelines reflect the established view of the scientific community with the following recommendations:

Guideline A: Any significant deterioration in graft dysfunction should be investigated using appropriate diagnostic tools and, if possible, therapeutic interventions should be initiated.

Guideline B: Any new onset and persistent proteinuria of 0.5g/24h should be investigated and therapeutic interventions should be initiated.

Premature death with a functioning graft is the most prevalent cause of graft loss in non-censored graft survival analysis (Ponticelli et al 2000). The main difficulties that arise with analysis of death-censored long-term graft survival are that not all failed grafts are studied histologically and that, in those which are, more than one condition may co-exist within the same graft. Data from a recent study of 197 failed grafts after the first transplant year showed CAN to be the leading cause of failure with almost 50% of cases being attributed to it (death with a functioning graft was second most common with 35% of cases attributed to this). The same study reported the death-censored projected graft half-life to be 31.1 years (Ponticelli et al 2002).

The differential diagnoses of chronic graft dysfunction, which can only be confirmed following histological evaluation, are as follows:

- chronic allograft nephropathy

- transplant glomerulopathy

- acute rejection (can occur at any time)

- calcineurin inhibitor nephrotoxicity

- transplant renal artery stenosis

- ureteric obstruction

- recurrent or de novo renal disease

'Chronic allograft nephropathy' describes the structural and functional changes in the graft that cannot be explained by other causes outlined above. It is characterized by a variable but usually progressive decline in GFR over months or years (Paul 1993) and up to 28% of patients will present with >0.5g proteinuria /24h (Massy 1996 et al). Clinical distinction of CAN from other conditions is not possible and requires a diagnostic biopsy specimen with subsequent histological analysis. The presenting features of other conditions may be similar to those seen with CAN e.g. proteinuria is evident in the majority of cases of transplant glomerulopathy, therefore the use of such criteria lack sensitivity or specificity. Indeed events such as late acute rejection episodes may occur without clinical symptoms or signs and are only picked up during routine follow-up.

Histopathological features of CAN

Individual histopathological findings in CAN are not specific although in combination a pattern of consistent changes is seen. These include graft vessel arteriosclerosis, glomerular lesions and glomerular sclerosis, infiltration with macrophages and lymphocytes, peritubular capillary basement membrane multilayering (seen under electron microscopy), interstitial fibrosis and tubular atrophy.

Graft atherosclerosis consists of concentric intimal thickening that affects large arteries and arterioles and is often accompanied by a moderate degree of infiltration of the vessel wall with macrophages and lymphocytes. The intimal thickening is thought to result from the migration of (myo)fibroblasts from the media to the intima, followed by local proliferation and deposition of extracellular matrix proteins. In vitro study of endothelial cells challenged by allogeneic lymphomonocytes shows they can promote the transdifferentiation of the functional phenotype of smooth muscle cells' from contractile to synthetic myofibroblastic phenotype (Amore et al 2001).

The glomerular lesions of CAN are variable and include collapse of the glomerular tuft, glomerular hypertrophy, mesangial matrix expansion and focal glomerulosclerosis. Transplant glomerulopathy is described as a lesion where there is enlargement of the glomeruli with swelling of the endothelial and mesangial cells, infiltration of the glomeruli with mononuclear cells, mesangial matrix expansion and widening of the subendothelial zone with interposition of mesangial cells and matrix (Hamburger et al 1964, Olsen et al 1992). In most grafts with CAN a non-diagnositc pattern of immunoglobulin deposition is seen. (Paul 1999).

The composition of extracellular matrix proteins deposited in CAN include tenascin, the extradomain A (EDA) isoform of cellular fibronectin, collagen type IV, laminin, heparin sulphate, decorin and collagen III.

CAN can be categorised in accordance with the Banff 97 working classification of allograft pathology to give more detail regarding the histological findings (Solez et al 1993, Racusen et al 1999)(see table below). CAN has been graded by chronic tubulointerstitial changes with or without specific vascular changes suggesting chronic rejection. In the lesion scoring scheme, chronic vascular lesions are scored as percentage arterial narrowing of the luminal area by fibrointimal thickening and using this system it is possible to artificially define grafts as showing 'chronic interstitial rejection' or 'chronic vascular rejection'. It has been shown that these two groups have different risk profiles with potential prognostic implications (Sijpkens et al 2001). A recent study of protocol biopsies taken at three months post-transplant confirm that patients who show signs of CAN with vasculopathy have a poorer 10 year graft survival than patients with biopsies showing CAN without vasculopathy (Seron et al 2000).

As stated previously, one of the possible pitfalls with the analysis of graft biopsy specimens is the existence of two or more conditions within the same graft as the presence of co-morbid pathology will not allow the clinician to confirm graft dysfunction to be entirely related to CAN.

Banff 97 diagnostic categories for renal allograft biopsies (Racusen et al 1999)

1. Normal

2. Antibody mediated rejection. Rejection demonstrated to be due, at least in part, to anti-donor antibody.

   A. Immediate (hyperacute)

   B. Delayed (accelerated acute)

3. Borderline changes: 'Suspicious' for acute rejection

4. Acute/active rejection

   Grades IIA (less severe), IIB, IIA(includes vascular rejection), IIB and III.

5. Chronic/sclerosing allograft nephropathy

Grade I (mild)	Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic rejection.
Grade II (moderate)	Moderate interstitial fibrosis and tubular atrophy (a) or (b).
Grade II (severe)	Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b).

6. Other

- Post transplant lympho-proliferative disorder

- Non-specific changes

- Acute tubular necrosis

- Acute interstitial nephritis

- Cyclosporin or tacrolimus associated changes, acute or chronic

- Subcapsular injury

- Pre-transplant endothelial injury

- Papillary necrosis

- De novo glomerulonephritis

- Recurrent disease

- Pre-existing disease

- Viral infection

- Obstruction/reflux, urine leak

- Other

In addition to the above, quantitative criteria exist for scoring

• tubulitis,

• intimal arteritis,

• mononuclear cell interstitial inflammation,

• early allograft glomerulitis,

- interstitial fibrosis,
- tubular atrophy,
- allograft glomerulopathy,
- mesangial matrix increase,
- vascular fibrous intimal thickening and
- arteriolar hyaline thickening.

Protocol biopsies

Protocol biopsies are performed on patients with functioning renal grafts to provide tissue for examination which gives both clinically relevant information that may affect patient management as well as being a valuable tool to the researcher. Serial protocol biopsies may be performed during the lifetime of the graft at time-points decided on by individual transplant centres. Some institutions elect not to perform protocol biopsies because it is a non-therapeutic intervention with potential patient morbidity. Non-protocol biopsies are performed when there is clinical indication e.g. deterioration in graft function.

Debate exists about the timing and number of protocol biopsies that should be performed when following up a renal transplant recipient as concerns remain about their reliability (Seron et al 2002). These specimens allow for the detection of disease which is not yet manifested clinically and it has been shown that some degree of CAN is present in 25% of patients at three months and 50% of patients at 2 years post-transplant in whom there had been no adverse clinical events (Legendre et al 1998).

The use of protocol biopsies relies on being able to identify histological parameters that may be used as primary efficacy variables. Intimal thickening and interstitial widening have been shown to progress rapidly over the first four months following implantation and slowly thereafter and may have a utility in this area (Moreso et al 2001). Early tubulointerstitial damage seen at three months profoundly influences graft survival beyond 10 years and fibrointimal thickening of small arteries and interstitial fibrosis at three months independently predict graft loss and decline in renal function (Nankivell et al 2001). Furthermore is has been proposed that protocol biopsies will have a use in the design of clinical trials aimed to prevent CAN and may allow a reduction of sample size and the timing of follow-up (Seron et al 2000).

In biopsies taken from grafts where a clinical indication exists at a time of six months or later following implantation the prognostic effect of arterial wall thickening is not

seen (Freese et al 2001). This study highlights that the cohort of patients who have protocol biopsies (i.e. all patients under follow-up in certain centres) is different to the patient group in whom clinical manifestations of graft damage have prompted the taking of a biopsy.

Whilst accepting that the use of both protocol and clinically-indicated biopsy specimens is the most common means of obtaining human renal allograft tissue to study there are two possible pitfalls here. First are the technical considerations such as variability in terms of technique and processing of the sample. To a large extent these variables can be minimised by the use of standardised procedures for the handling of tissue. The second pitfall to consider is whether such biopsy specimens are truly representative of the whole organ. This problem is highlighted by the study of CD31 immunohistochemical staining within human breast cancers which showed that microvessel counts from biopsies did not correlate with the microvessel count obtained from a cross-section of the whole gland (Jacobs et al 1998, Marson et al 1999). Similar investigation of observer variability and reproducibility of biopsy specimens form part of the work of this thesis.

The use of animal models of disease can provide valuable information and these will be briefly examined in the next section.

Animal models of CAN

Study of progression of CAN may be optimised by the development of animal model systems.

A rat model of CAN exists using uninephrectomised Lewis rats as recipients and Fisher 344 kidney allograft donors (Nagano et al 1997). A cardiac model of chronic rejection in mice (heterotopic cardiac mouse transplantation of CBA strain into C57BL/6) was able to mimic the clinical characteristics seen in humans (Koglin et al 1999).

A mouse model of CAN is under development in our research group. No tissue was available for study during the time allotted to this study but will provide an excellent method for undertaking investigation of the role of the endothelial cell and macrophage in the development of CAN. The model has been developed in the United States and Canada (Mannon et al 1999). One strain of mice e.g. B.10.A will be used as donors and kidneys will be transplanted into a different strain of recipient mice e.g. B.10.BR with only one allele difference so that acute rejection episodes are avoided. As non-rejecting isograft controls, kidneys will be transplanted between litter mates. The donor kidney, ureter and bladder will be harvested en bloc and transplanted onto recipient abdominal aorta, inferior vena cava and bladder. The right native kidney will be removed at the time of the transplant and a left nephrectomy performed seven days later. Renal function will be assessed at various time points following transplantation and will include proteinuria, serum creatinine and creatinine clearance. Various histological assessments of the kidneys will be made following sacrifice of the mice.

Risk factors and prognostic indicators of CAN

Until molecular understanding of the pathogenesis of CAN is better defined and novel therapeutic strategies are under development, any advances in long-term graft survival will come from the optimisation of donor and recipient factors. Donor or host age, gender and co-morbidity are difficult to optimise whilst there is a premium on finding sufficient numbers of matched donors as is the case in this country (UKT 2002). Refinements of existing and newer immunosuppressive therapy will be considered in a later section.

Previously this progressive decline in graft function had been termed 'chronic rejection' reflecting the belief that it resulted from a continuous immunological aggression of the host against the allograft. The term chronic allograft nephropathy has evolved to reflect the fact that both allo-antigen dependent and allo-antigen independent mechanisms may participate in the development of graft dysfunction (Ponticelli 2000). It is likely that CAN develops as a result of a combination of both immunological and non-immununological factors.

Immunological Factors – Alloantigen-dependent

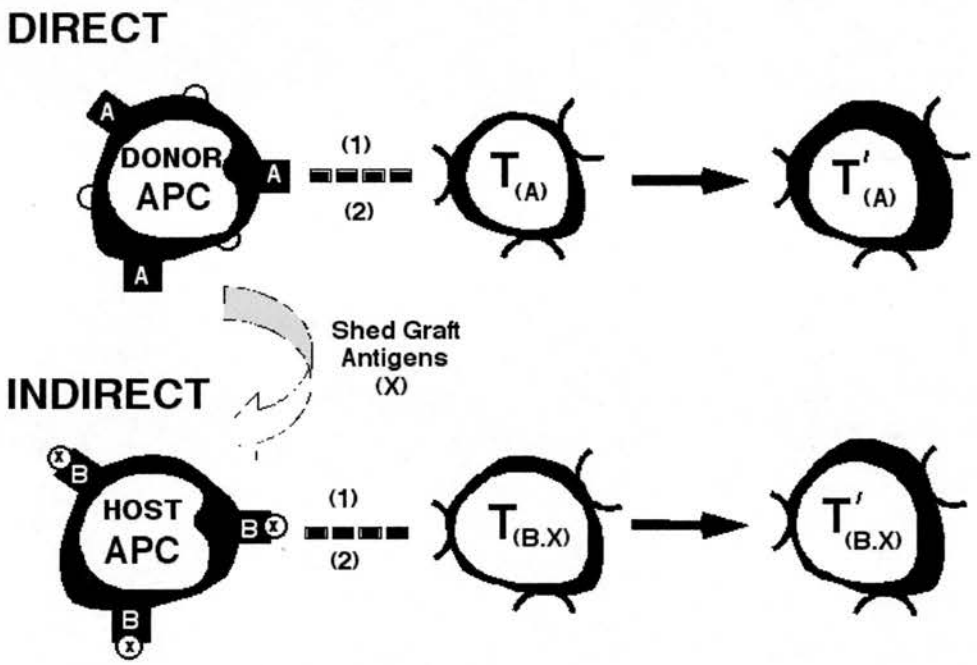
Chronic rejection (CR) is an antigen-dependent immune process leading to the lesions observed in CAN. Allotransplantation of organs results in activation of the immune system by alloantigen-dependent processes and recognition of foreign antigens on cells of the graft results in activation of specific immune responses (Joosten et al 2003). As such, all recipients of an allogenic kidney graft should receive life-long immunosuppressive medication (see below).

Several studies have shown that patients who experience episodes of acute rejection (especially those occurring after the first 4 months) have a higher incidence of CAN and graft loss (Shishido et al 2003, Massy et al 1996, Baboolal et al 2002).

The Collaborative Transplant Study (CTS) data presented at the 17th World Congress of the Transplantation Society in 1999 shows a strong association of the number of HLA-A+B+DR mismatches with graft survival rate. HLA (human leucocyte antigen) matching has been shown to have a beneficial effect on long-term graft survival and graft half-life (Opelz et al 1999) and the best results were observed with HLA-identical sibling donors (Teraski et al 1995). After organ transplantation the immune system of the recipient encounters various foreign antigens in the graft. All donor antigens that differ from those of the recipient evoke an immune response if presented to the immune system. A possible explanation as to why mis-matching may lead to CR is that HLA incompatibility increases the immunogenicity of the graft and triggers a continuous subclinical aggression against the allograft, eventually resulting in its progressive loss. Antigen presentation may follow one of two pathways – direct and indirect.

Direct antigen presentation

Donor alloantigens are presented to recipient T-cells by donor-derived antigen presenting cells (APCs). This will mainly result in the activation of type-1 responses and thus activation of CD8+ cytotoxic T-cells.



Indirect antigen presentation

Infiltrating recipient-derived APCs present donor alloantigens (after their uptake) to recipient T-cells activating CD4+ T-cells. It has also been suggested that indirect presentation results in the production of alloantibodies by B-cells (Joosten et al 2003)(see below).

Direct presentation is thought to be mainly involved in acute rejection, whereas indirect presentation is suggested to be more important for CR (Waaga et al 2000). Induction of an immune response after recognition of antigens by either method requires a second or co-stimulatory signal between the APC and T-cell e.g. the

CD40-CD154 pathway. These co-stimulatory signals are essential for the induction of immune responses and thus for transplant rejection and their blockade in allograft models improves graft survival (Sayegh et al 1998).

Immune activation can be initiated not only by the recognition of specific antigen ('acquired immunity') but also by the recognition of non-specific injury ('innate immunity'). Such non-specific injury that occurs during transplantation (e.g. ischaemia) initiates inflammation by recruitment of lymphocytes, macrophages and dendritic cells. This triggers the production of the co-stimulatory pathways that stimulate T-cells which are central to the process of CR (Lu et al 1999). Furthermore, studies in rats have shown brain death of the donor up-regulates various lymphocyte and macrophage derived cytokines on somatic organs thus increasing their immunogenicity (Takada et al 1998).

The role of alloantibodies in CAN

Alloantibodies seem to be important mediators of CR. Antibodies that have been recognised as significant are those against HLA, perlecan, collagen, mesangial cells and endothelial cells (Joosten et al 2003). Renal transplant recipients with anti-HLA antibodies have been found in 12-60% of recipients and these patients were 5 to 6 times more likely to develop CR (Sumitran-Holgersson et al 2001, McKenna et al 2000). Antibodies against endothelial cells have been found in renal transplant recipients before and after transplantation (Ball et al2000). In a cohort of patients with at least one failed graft, 14% had endothelial cell-specific antibodies compared with 3% for recipients with stable function (Perrey 1998). Endothelial cell activation induced by antibodies could also contribute to increased extravasation of allo-immune immunocompetent cells to facilitate rejection.

Deposition of immunoglobulins in the graft is transient and difficult to detect but since antibody binding results in deposition of complement, the more stable complement split product C4d can be used as a marker for humoral rejection. In late allograft biopsies C4d deposits were detected in one third of those studied. However in kidneys that already had advanced lesions of CR, glomerular basement membrane lesions or allograft arteriopathy, C4d deposits were found in 60% of biopsies (Mauiyyedi et al 2001). The high percentage of C4d positivity implies a role for antibodies in the pathogenesis of these lesions. The presence of C4d in biopsies correlated with anti-donor HLA antibodies; 88% of patients with C4d deposits also had these antibodies in their circulation (Mauiyyedi et al 2001, Lederer et al 2001).

Non-alloimmune Factors – Alloantigen-independent

• Advanced donor age – An analysis using the UNOS Scientific Renal Transplant Registry (Terasaki et al 1997) demonstrated a strong effect of donor age on long-term outcome and graft half-life and this was attributed to a relative deficiency in the number of nephrons with regard to the recipient's metabolic demands.

• Glomerular hyperfiltration – It is hypothesised that, as a consequence of decreased renal mass that occurs after transplantation, ensuing glomerular hyperfiltration in the remaining nephrons provokes progressive glomerulosclerosis leading to graft failure (Sola et al 2002).

• Delayed graft function – Transplants with prolonged ischaemic exposure and delayed graft function experience inferior long-term outcome (Shoskes et al 1996).

• Proteinuria – Patients who subsequently lost their grafts to chronic rejection more commonly had proteinuria (>0.5g/24hr) in the first 24 months after transplantation and that proteinuria was an independent risk factor for chronic rejection (Massy et al 1996).

• Arterial hypertension – The degree of hypertension during the first 24 months after transplantation has been shown to be a risk factor for developing chronic rejection using univariate analysis, but the effects of hypertension were not independent of other risk factors in multivariate analysis (Massy et al 1996). Although it is likely that adequate control of the hypertension associated with chronic rejection will improve graft outcome this is yet to be proven in controlled trials (Raine et al 1995).

• Hyperlipidaemia – Hypertriglceridaemia (>200 mg/dl) has been shown to be an independent risk factor for chronic rejection and that this effect was in turn independent of pre- and post-transplant diabetes mellitus (Massy et al 1996). Graft biopsies from patients with elevated cholesterol levels often display more severe

chronic damage than biopsies from patients with lower cholesterol levels, although this has not been shown in all studies (Cardinal et al 2002, Paul 1999).

• CMV infection – Cytomegalovirus disease has been shown to be associated with early graft dysfunction (Tong et al 2002) and to be an independent risk factor for CAN (Sola et al 2003). Graft outcome and loss has not been shown to be primarily attributed to CMV infection but rather determined by acute rejection episodes and the development of CAN (Kashyap et al 1999). CMV disease is however associated with early graft dysfunction (Tong) and is preceded by treated episodes of acute rejection in 65% of cases. CMV infection is probably associated with CAN suggesting that an increase in viral load leads to a higher risk of developing CAN (Sola et al 2003).

Proposed pathogenesis of CAN

Inflammatory response to graft injury

Renal injury results in structural damage and impairment of function. A stereotypical inflammatory response occurs following renal injury and comprises:

- influx of lymphocytes, monocytes and macrophages
- proliferation of fibroblasts
- deposition of extracellular matrix material
- scar formation
- tissue restoration

This tissue remodelling is the beneficial effect of the inflammatory cascade but it also causes inhibition of cell proliferation and apoptosis with cell loss and this is potentially deleterious to the affected tissue. Furthermore, fibrosis and scar formation results in disruption or loss of the normal microvascular skeleton which is likely to lead to further tissue hypoxia.

The macrophage has an important role in the outcome of inflammation (Morrissette et al 1999) and significant increases in macrophages and myofibroblasts occur in early renal biopsy specimens in patients who later develop CAN (Pilmore et al 2000). Macrophages may be stimulated by both humoral and cellular immunity and non-immune mechanisms and once activated their effects are pleotrophic (Croker et al 1996). These are summarised on the next page.

Macrophage activation

• T-cells and macrophages have a reciprocal relationship: T-cell derived IL-2 and IFN-γ can stimulate macrophage activation whereas macrophages play a role in T-cell activation by producing T-cell activators such as IL-1 and as antigen presenting cells.

• Intrinsic renal cells may directly stimulate macrophages e.g. glomerular endothelial cells produce monocyte chemoattractant protein 1 (MCP-1).

• Antibody and complement binding and activation stimulate macrophages.

• Autocrine and paracrine loops exist for macrophage activation. MCP-1 and IL-1 are produced by macrophages which also have receptors for these cytokines and are stimulated by them.

Effect of macrophage activation

• Macrophages produce a variety of factors which affect blood vessels in the short and long term e.g. platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), nitric oxide (NO), thromboxane, transforming growth factor-β (TGF-β).

• Interstitial cells proliferate and produce matrix proteins in response to macrophage products.

• Macrophage activation in quiescent renal allografts augments rejection (Nagano et al 1997) and the presence of glomerular macrophage infiltration predicts a significantly worse graft outcome (Ozdemir et al 2002). This may be as a result of deleterious rather than reparatory cell loss and there is in vitro evidence shows that macrophages can induce endothelial cell apoptosis (Lang et al 1993, Lang et al 2003 unpublished).

Ischaemia-reperfusion injury and donor brain death can induce or amplify the immune response via the production of reactive oxygen species, pro-inflammatory mediators and growth factors, increased expression of adhesion molecules and upregulation of both HLA class-I and class-II molecules (Stoica et al 2002, Szabo et al 1998, Takada et al 1998).

After ischaemia-reperfusion tissue injury, interleukins 12 and 18 induce interferon-γ with subsequent upregulation of MHC class-I and class-II antigens and activation of the endothelium, triggering costimulatory molecules and T cell facilitation (Szabo et al 1998, Daemen et al 1999). This, together with tubular epithelial cell apoptosis (Chien et al 2001, Daemen et al 2002), results in increased recruitment of inflammatory cells and inflammatory processes which can further facilitate alloantigen dependent processes described above. Thus these can be considered as progression factors for CAN. Cytokine excess may be a contributory factor. It is proposed that repetitive injury over a short period of time results in excessive production of fibrogenic cytokines including transforming growth factor β (TGF-β) (Cuhaci et al 1999) and platelet derived growth factor (PDGF). These cytokines promote excessive deposition of extracellular matrix proteins with associated fibrosis as is seen in CAN.

Vascular injury

The most widely described hypothesis to explain CAN is that vascular injury is the trigger for the inflammatory response to injury detailed above. Indeed vascular rejection correlates most strongly with graft prognosis (Van Saase et al 1995). Furthermore, studies of grafts with CAN show different risk profiles for interstitial versus vascular types of chronic rejection with biopsies showing CAN with vasculopathy have a 10 year graft survival that is half that of CAN without vasculopathy (Sijpkens et al 2001, Seron et al 2000).

Endothelial cell damage results in a combination of increased endothelial permeability, increased exposure of host antigens and a prominent inflammatory response by endothelial cells themselves (Akyurek* 1995). Angiogenesis is likely to be stimulated as a result of hypoxia following endothelial cell loss. A recent study has demonstrated increased vascularity in biopsies from grafts with signs of CAN versus both acute rejection and normal kidney. The increase was observed in association with the development of interstitial fibrosis and poor outcome (Ozdemir et al 2002). VEGF has been shown to be up-regulated in CAN and its expression correlated with macrophage expression (Pilmore et al 1999).

Vessel wall proliferation and vascular sclerosis result from growth regulating cytokines produced by endothelial and smooth muscle cells in response to previous vessel wall injury, resulting in activation of self-amplifying autocrine and paracrine activation cascades. If vessel wall lesions are considered to be the primary lesions in CAN, the tubulointerstitial and glomerular lesions that are seen may be considered as secondary or collateral to these vascular lesions.

Conflicting evidence exists which suggests that CAN emerges as a result of disruption of the graft vessel lymphatics (Demetris et al 1997) and that obstruction of the lymphatics that drain the vessel wall will cause the vessel wall lesions as observed in CAN (Solti et al 1991).

It is likely that CAN emerges as a consequence of primary vascular or lymphatic lesions or a combination of the two.

Premature senescence and donor age

Chronic allograft nephropathy may represent the exhaustion or senescence of graft endothelial cells or tubular cells. Senescence can best be thought of as 'wear and tear' of cells. Somatic cells in culture can undergo only a limited number of divisions after which they become senescent resulting in reduction in processes such as energy generation and replication to shut down. Histologically this is characterised by membrane damage and cellular atrophy and the process may be accelerated by immune-mediated and/or non-immune injuries (Halloran et al 1999). In the development of CAN it could therefore be argued that the fibrosis that is observed may be as a result of a loss of regulatory control of inflammation and healing by senescent graft cells which are ultimately exhausted.

As described above, (Terasaki et al 1997) advanced donor age correlates with inferior long-term graft outcome in some studies (Alexander et al 1994), although conflicting hypotheses have been presented to explain this, including the concept of senescence or that increased donor age and reduced renal mass lead to hyperfiltration and glomerulosclerosis (Brenner et al 1992). Micropuncture studies in rats have shown glomerular hypertension to exist in kidney grafts with CAN (Kingma et al 1993). However in the same study glomerular hypertension in syngeneic grafts did not result in CAN. This suggests that glomerular hypertension is a permissive or progression factor in the pathogenesis of CAN rather than a direct cause. This statement is further supported by the results of two studies that failed to demonstrate an indirect effect of donor age on graft outcome. An equivalent risk of graft failure was demonstrated when comparing donors aged 75 years from the period 1994-1997 with donors aged 30 years from the period 1983-1990 (Roodnat et al 1999). If the pathogenesis of CAN is to be assumed to be unchanged between these two time points then it is impossible to support a hypothesis that an ageing donor kidney with reduced renal mass is solely responsible for the development of CAN and that the effects of improved immunosuppression and control of immune mediated reactions have a greater importance than other risk factors. The second study (Kerr et al 1999) demonstrates that excellent results from older donors can be obtained when they are

HLA-related living donors. Disproportion between donor and recipient size has not conclusively been shown to alter graft outcome.

Immunosuppression and CAN

Until the 1980s standard immunosuppression consisted of corticosteroids and azathioprine, complemented in some centres with the prophylactic use of anti-lymphocyte antisera in the first few post-transplant weeks (Paul 1999). The first two agents are still regularly of use in current practice together with additional newer treatments but caution must be exercised when interpreting data regarding the impact on the development of CAN.

Unfortunately there is a relative lack of randomised trials with sufficient population sizes and robust follow-up information to fully assess the impact of various immunosuppressive drug regimes on long term outcome. Reports from both the National Institute for Clinical Excellence (NICE) and British Transplantation Society (BTS) from the last two years concluded that there are insufficient data to permit specific recommendations regarding immunosuppressive therapy and that all currently available immunosuppressive drugs should remain freely available for use by transplant centres. Formal randomised controlled trials can be difficult to construct owing to heterogeneity of patient population and relatively small size of sub-groups as well as the significant and variable side effect profile of the drugs. Furthermore, the impact of newer treatments such as mycophenolate mofetil on the development of chronic allograft nephropathy will only become apparent over the next few years when longer follow-up periods are available.

As well as the maintenance regimen of oral immunosuppressive therapy many patients receive anti-T cell antibody therapy peri-operatively e.g. anti-thymocyte globulin (ATG), and the impact of these drugs on development of CAN has not been extensively reported.

As such the following section will focus on the following immunosuppressive agents:

- corticosteroids
- calcineurin inhibitors
- azathioprine and mycophenolate mofetil
- sirolimus

Corticosteroids

In the Collaborative Transplant Study database the five year graft survival rates were significantly better in patients who had been switched from CsA, steroids and azathioprine to steroid-free maintenance immunosuppression with CsA, with or without azathioprine (Opelz et al 1994, Opelz et al 1995). This suggests that the detrimental effect of corticosteroids exceeds their potential benefit on long term graft outcome, although only 10% of the patients reported were treated with the steroid free regimens and it is likely that these were low risk patients. More recently it has been shown that steroids can be safely withdrawn in patients treated with mycophenolate mofetil (Grinyo et al 1997) but with no long term data to assess its impact on chronic allograft nephropathy.

Other studies have attempted to address this issue of steroid withdrawal and its effect on short and long term graft outcome without arriving at a consensus decision. As such the use of corticosteroids (both dose and duration) will continue to be a matter for each individual transplant centre to decide upon until data becomes available from a large, multi-centre, randomised controlled trial with long follow-up.

Calcineurin inhibitors

Cyclosporine

During the 1980s the introduction of the calcineurin inhibitor cyclosporine (CsA) resulted in a decrease in the incidence of acute rejection episodes and an improved one year graft survival rate. However United Network for Organ Sharing (UNOS) data (Gjertson et al 1992) demonstrated that it did not affect the rate of graft loss occurring after the first year (Terasaki et al 1992). It is likely that the immunosuppressive efficacy is offset by its nephrotoxic side effects (Myers et al 1984) or that inadequate doses have been prescribed as a result of concerns regarding its nephrotoxicity (Burke et al 1994). A third possibility is that the drug may have no effect on chronic allograft nephropathy.

When referring to the United Network for Organ Sharing (UNOS) data the presence of variables affecting graft survival other than immunosuppression makes evaluation of the impact of CsA introduction on long term graft outcome more difficult. There were a number of differences between the time periods assessed (1975-79 pre-CsA versus 1985-90 with CsA). The expansion in the numbers of transplant centres away from a smaller number of expert centres may be an important variable as well as changes in clinical practice experienced. In the past most transplant recipients were young, without significant comorbidity and received the kidney from a young donor. More recently, recipients of renal transplants have been older and with comorbid conditions such as diabetes, cardiovascular disease or other systemic diseases (Ponticelli 2000) and this will have affected graft survival in the time frame of the data.

The effect of the introduction of cyclosporine therapy in the 1980s on the treatment of patients with renal transplant has been described together with concerns over biases between the two time periods compared in the UNOS study. This may have skewed results to support a hypothesis that cyclosporine therapy confers no long term benefit on graft outcome. The most recent UNOS data now reports an increase

in cadaveric graft half life to 10.4 years (from 7.8 years in cyclosporine treated patients between 1985 and 1990) for renal transplants performed between 1995 and 1996 (Ponticelli 2000). By comparing this figure with experience from other controlled and uncontrolled studies from recent years where patients have been receiving cyclosporine it can be seen that excellent results can be obtained. A study of 632 cyclosporine treated patients showed a graft half life of 19.9 years with a death censored half life of 24.8 years (Montagnino et al 1997). A 12 centre collaborative trial which investigated three different modalities of cyclosporine administration in cadaveric renal transplant recipients showed mean graft half lives of 16.4, 12.6 and 19.0 for single, double and triple therapy respectively (Tarantino et al 1998). A study of young renal transplant recipients (<50 years of age) treated with cyclosporine reported a graft half life of 21.8 years and a review of the data of the Collaborative Transplant Study showed a half life of more than 17 years for first cadaveric grafts treated with cyclosporine based triple therapy (Opelz et al 1995). These studies suggest that certain groups of cyclosporine treated renal transplant recipients have graft survival far in excess of the figures quoted in the UNOS studies. Furthermore the preparation of cyclosporine used in most transplant centres has changed to a microemulsion in recent years with an improved pharmacokinetic profile (Goel et al 2002). Chronic allograft failure (CAF), acute rejection rates and graft survival have been compared between different preparations of cyclosporine and with tacrolimus. Both tacrolimus and cyclosporine microemulsion are associated with a significantly better adjusted CAF-free survival at 4 years (Meier-Kriesche et al 2002) with improved graft survival compared with the older conventional cyclosporine formulation (Boots et al 2001).

Despite the fact that cyclosporine is undoubtedly a nephrotoxic agent there is evidence that when administered appropriately a deleterious effect on long term graft outcome may be avoided (Burke et al 1994). In contrast a prospective trial of conversion from cyclosporine to azathioprine after the third month post-transplantation showed a trend toward better patient survival, graft survival and longer uncensored half life at 13 year follow-up in the group converted to azathioprine but without reaching statistical significance. Chronic allograft

nephropathy was present in 12% of cyclosporine treated patients compared with 8% of the azathioprine-treated group (Paul 1999). Another study confirms short term patient and graft survival have improved with the use of cyclosporine but that this effect is not maintained in the long-term (Marcen et al 2001).

Reduction in the dose of calcineurin inhibitors in patients with histological evidence of nephrotoxicity resulted in a sustained improvement of renal function (Pilmore et al 2002) and such an intervention was safe, well tolerated and associated with minimal risk of acute rejection (Weir et al 2001).

Tacrolimus

Tacrolimus (or FK-506) is another calcineurin inhibitor and offers an alternative to cyclosporine. Both this and CsA can cause histological and functional renal impairment that is difficult to distinguish from chronic allograft nephropathy (Myers et al 1984, Connolly et al 1994).

In a randomized open-label trial comparing cyclosporine A with tacrolimus, one year graft and patient survival rates were similar although the tacrolimus-treated group showed a significant reduction in the incidence and severity of biopsy proven acute rejection episodes (Pirsch et al 1997).

The effect of conversion to tacrolimus from cyclosporine on long term graft outcome and chronic allograft nephropathy is unclear and conflicting evidence has been presented. UNOS kidney transplant registry data shows a significantly longer projected graft half life in patients treated with tacrolimus compared with those treated with cyclosporine A (Gjertson et al 1995). With the addition of supplementary information these data were further confirmed showing that tacrolimus improves graft survival and half life (Katznelson et al 1996). However these data have been contrasted with analysis of the Collaborative Transplant Study (Opelz et al 1999) which show that immunosuppression with tacrolimus results in graft outcome that is comparable to, but not significantly better than, conventional cyclosporine A. It is important to consider that these studies have compared

tacrolimus with non-microemulsion CsA ('Sandimmune')which has been shown to be less efficacious than the newer microemulsion CsA ('Neoral'). More recently a study with longer follow-up (6 years) has shown that patients receiving tacrolimus had significantly greater 6 year graft survival and a higher projected graft half life than those receiving cyclosporin microemulsion (Jurewicz et al 2003). A smaller Italian study further supports conversion to tacrolimus to be safe and efficacious (Pretagostini et al 2001) and a randomised trial comparing cyclosporine microemulsion to tacrolimus revealed an association with increased allograft fibrosis and significantly higher serum low-density lipoprotein cholesterol levels in the cyclosporine treated group (Murphy et al 2003).

The difference in effect of these two drugs may be due to differing expression of the pro-fibrotic cytokine transforming growth factor β (TGF-β). A prospective study of 51 patients randomised to cyclosporine or tacrolimus revealed a ten fold increase in TGF-β mRNA in protocol biopsies from the cyclosporine treated group which was associated with interstitial fibrosis (Baboolal et al 2002). The same study concluded that calcineurin inhibitors (and acute rejection episodes) were the most important factors contributing to early renal structural injury.

Azathioprine

The Collaborative Transplant Study registry showed no difference in the estimated half life of kidney transplants in patients treated with CsA and corticosteroid only compared with CsA, corticosteroid and azathioprine, suggesting that azathioprine does not improve long term outcome (Opelz et al 1995). These findings have been repeated in other studies and meta-analyses.

Mycophenolate mofetil (MMF)

Studies addressing whether the use of mycophenolate mofetil affects long term graft survival, patient survival or the development of chronic allograft nephropathy are yet to be completed as follow-up times are too short. It is known that MMF in conjunction with CsA decreases the incidence of acute rejection in the first six months after transplantation by approximately 50% compared with placebo or azathioprine (European MMF trial). There are also studies which have looked at grafts treated with MMF at three years post-transplantation which suggest better graft survival than those treated with azathioprine (Ojo et al 2000) (Meier-Kriesche et al 2001a) and that a reduction in the risk of chronic allograft nephropathy occurs independently of acute rejection episodes (Meier-Kriesche et al 2001b).

In patients who have chronic, progressive deterioration of renal function after transplantation the addition of MMF with reduction of cyclosporine dosage is well tolerated and results in a short term improvement in renal function (Weir et al 1997). This improvement in renal function with the use of MMF to 'cover' dose reduction of cyclosporine has been described elsewhere and provides stronger immunosuppression than by using azathioprine (Jain et al 2001). Furthermore it has been shown that the improvements are achieved irrespective of changes in blood levels of cyclosporine A (Gonzalez Molina et al 2002). Adverse effects associated with MMF include gastrointestinal upset and, in combination with tacrolimus, neutropenic sepsis (Jain et al 2001, Metcalfe et al 2002).

MMF acts to inhibit the de novo synthesis of purines which are crucial to cell cycling of T and B cells. It therefore blocks the clonal expansion of B and T cells preventing antibody production and the generation of cytotoxic T cells as well as other effector T cells (Mele et al 2000). In cardiac transplantation patients treated with MMF produced fewer anti-vimentin antibodies than patients treated with azathioprine (Rose et al 2002). These data suggest that MMF prolongs graft survival due at least in part to a decrease in the production of alloantibodies. However as mentioned above, long term follow-up data is required to fully assess the impact of MMF on chronic allograft nephropathy.

Sirolimus (rapmycin)

The recent introduction of the potent immunosuppressive agent sirolimus has afforded an opportunity to develop a regimen designed to maximize prophylaxis of early acute rejection without drug-induced nephrotoxicity. Sirolimus, like cyclosporine, is a macrolide with potent antilymphocyte activity. Unlike cyclosporine it complexes with a different cytosolic immunophilin (FKBP12), resulting in suppression of cytokine driven T cell proliferation by inhibiting progression from the G_1 to the S phase of the cell cycle.

The three approaches of CNI elimination, avoidance or substitution have been recently attempted each with the goal of reducing CNI induced nephrotoxicity. The term CNI Avoidance implies a de novo immunosuppressive regimen without a CNI and employs the use of one to three maintenance agents. The term CNI Substitution describes the practice of using an alternative to a CNI drug in a maintenance regimen. The many combinations of maintenance regimens now available can make direct comparisons between initial regimens problematic. A potential penalty for such CNI free immunosuppression is, of course, increased rates of acute (and perhaps chronic) rejection, which would obviate the benefit of alternative regimens.

Early cyclosporine withdrawal using sirolimus has been shown to be followed not only by an improvement in renal function, but also by a reduction in the rate of progression of chronic pathologic allograft lesions and in the appearance of new cases of CAN (Ruiz et al 2003). This effect is especially important in the case of tubular and interstitial lesions, probably as a consequence of a reduction of CyA nephrotoxicity. It is possible that this beneficial effect might be responsible for better graft outcome after longer follow-up periods (Flechner et al 2003). This is supported by a smaller study (40 patients) which demonstrated fewer histological signs of chronic damage, especially vascular injury, in patients who had sirolimus immunosuppression after withdrawal of calcineurin inhibitor therapy at 3 months after transplantation (Stallone et al 2003). The use of sirolimus as a CNI sparing agent, rather than as part of CNI-free regimens, has a more limited use. In a study of

70 patients there were higher incidences of acute rejection and histological signs of CAN at 1 year in patients randomized to a CNI sparing regime (sirolimus, MMF and dose reduced tacrolimus) compared with a CNI free regime (sirolimus and MMF). A further study of 31 patients which compared CNI dose reduction and introduction of sirolimus as a CNI sparing agent demonstrated no functional, molecular or histological improvements at 1 year compared with a standard CNI based regime (Saunders et al 2003).

Rapamycin (sirolimus) has been shown to have actions that can disrupt angiogenesis. The P13K/Akt signalling pathway plays a critical role in regulating basic cellular functions including cell proliferation and angiogenesis and mTOR (mammalian target of rapamycin) is a downstream mediator in this pathway (Chan et al 2004). In ovarian cancer cells rapamycin has been shown to induce G(1) cell cycle arrest and inhibit expression of important cyclins (Gao et al 2004). Similarly it has been shown that the chronic myeloid leukaemia oncogene BCR/ABL induces VEGF gene expression and its transcriptional activator HIF-1 alpha through a pathway involving P13K and mTOR and that this expression is inhibited by addition of rapamycin and LY294002 (Mayerhofer et al 2004).

Rapamycin may also inhibit angiogenesis through an alternate pathway. Functional angiogenesis induced by fibroblast growth factor-2 is dependent upon the platelet derived growth factor receptor-alpha (PDGFRalpha)-p70S6K signal transduction pathway in mesenchymal cells. Rapamycin inhibits p70S6K and in a murine model has been shown to shut down the sustained expression of VEGF and hepatocyte growth factor (HGF). In each tumour tested, rapamycin constantly led to tumour dormancy via silencing of the PDGFRalpha-p70S6K pathway (Tsutsumi et al 2004).

Summary

Analysis of these studies confirms that there is no consensus as to the use of one particular immunosuppressive drug regimen compared to another. This decision will remain at the discretion of the individual clinician and transplant centre.

Novel treatment options

Currently there is no established maintenance immunosuppressive regimen that decreases the incidence of chronic allograft nephropathy in humans.

Experimental animal studies have shown that immune tolerance prevents chronic allograft failure; in skin and cardiac grafts by CD-28 and CD-40 blockade (Larsen et al 1996) and with T-cell co-stimulation blockade (see above) in renal allografts (Azuma et al 1996). There is, as yet, no evidence that this therapy will be feasible in humans.

Alternatives to immune modulation involve the use of non-immunosuppressive drugs such as somatostatin analogues (Hayry et al 1993), eicosanoid and platelet activating factor inhibitors (Hayry et al 1993), heparinoids (Aziz et al 1993), insulin-like growth factor antagonists (Hayry et al 1995), oestrogens (Lou et al 1996a) and nitric oxide donors (Lou et al 1996b). Angiotensin receptor blockers have also been tested (Furukawa et al 1996) as have angiotensin-converting enzyme (ACE) inhibitors (Benediktsson et al 1996) and one retrospective clinical study has shown ACE inhibitors to be beneficial in chronic allograft nephropathy (Barnas et al 1996). As described above, systemic hypertension and hyperlipidaemia post-transplantation should be treated appropriately (Raine et al 1995, Massy et al 1996) although there is no evidence to show that this will reduce the rate of graft loss.

The major problem facing future investigators is the design of trials with sufficiently large sample size, long follow-up periods and clearly defined end-points if these advances are to be converted into clinical benefit.

Suggested current management

Management of patients should take place in specialist, expert transplant centres. Avoidance of graft damage as a result of ischaemia-reperfusion injury and ensuring good HLA matching with consideration of preferential use of living related donors should optimise graft peri-operative variables.

Undertaking aggressive management of acute rejection episodes (at whatever time) to ensure complete reversal of graft function should be standard practice. When choosing maintenance immunosuppression regimen, minimizing the use of nephrotoxic agents (such as calcineurin inhibitors) and corticosteroids should be the aim. Consideration of the use of surveillance biopsies must also be employed.

The provision of organs for transplantation is a simple case of supply and demand with demand out-stripping supply in the UK at present (UKT). Most discussions of the supply-demand dichotomy focus on the supply side of the equation however attempts to diminish demand are just as important. Any course of action that prolongs the functional survival of already transplanted kidneys will diminish the demand for retransplantation. In fact, significant prolongation of live donor kidney transplant survival may obviate the need for some patients to ever enter the waiting pool for scarce cadaveric organs. There are also medical economic advantages to prolonged graft survival. Recent analyses have demonstrated that initial increases in the adjusted costs (compared to maintenance dialysis) of renal transplantation are eclipsed after 2 years of graft survival. Beyond this interval savings to health care systems begin to accrue. Therefore, prolongation of graft survival is beneficial to both individual recipients and those who fund health care (Flechner et al 2003).

This thesis adds to the body of knowledge about the development of CAN. Ultimately work in this field will result in improved management of CAN with the benefits described above.

Aims of the thesis

To determine changes in vascularity which occur in diseased human parathyroid tissue and during the development of chronic allograft nephropathy.

The aims are addressed sequentially:

1. To investigate changes in vascularity between normal parathyroid tissue, hyperplasia and adenoma and to seek an association with the endothelial cell mitogen VEGF.

2. To undertake reproducibility experiments of the methodology used to assess vascularity.

3. To validate the study of changes in vascularity in kidney disease with the use of renal biopsies.

4. To examine changes in vascularity that occur during the development of chronic allograft nephropathy and to seek an association with endothelial cell proliferation and macrophage infiltration.

Chapter 2: Materials and methods

In order to achieve the aims, five main studies have been performed described below. Methods involved in the studies will be described in this chapter.

1. Vascularity of normal parathyroid, hyperplasia and adenoma were compared using immunohistochemistry. The expression of VEGF protein and mRNA were investigated using immunohistochemistry, Western blotting and in situ hybridisation.

2. i) Observer variation and reproducibility of microvessel counts (MVCs) was compared between the two principal investigators.

   ii) The use of microvessel counts in renal biopsies was examined by comparing MVCs in kidney biopsies with cross section specimens from the same kidney.

3. Changes in vascularity, endothelial cell proliferation and macrophage infiltration between normal kidney and allograft nephrectomies performed because of chronic allograft nephropathy.

4. Sequential assessment of changes in vascularity, endothelial cell proliferation and macrophage infiltration during the development of chronic allograft nephropathy using allograft biopsies.

Similar methodologies have been adopted throughout the thesis based on obtaining specimens of human parathyroid glands and specimens of kidneys in the form of biopsies or excision specimens. Sections were immunohistochemically stained with antibodies to the CD31 elements of the endothelial cell, to VEGF and to proliferating cells (MIB-1 antibody to Ki-67). Assessments were then made by microvessel counting or by estimation of the percentage of cells staining. Western blotting and in situ hybridisation were only performed on parathyroid tissue to assess expression of VEGF.

Methodological details describing aspects of the method common to all studies, including histology and immunohistochemistry are presented first. This will be followed by sections corresponding to each study performed. Full details of materials used are described in the Appendix to this chapter.

CD 31 immunohistochemistry – APAAP method

Tissue sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and placed into deionised water. Antigen retrieval was achieved using protease (Protease XXIV, Sigma) at 37°C for exactly 20 minutes. Sections were loaded onto cover plates (Thermo Shandon) and DAKO serum-free protein block was applied for 30 minute incubation followed immediately with primary antibody (mouse monoclonal anti-human CD31, 1:20 dilution, DAKO) for 1 hr at room temperature without intervening wash. Sections were then washed with phosphate buffered saline (PBS) prior to incubation with secondary antibody (mouse immunoglobulins, 1:50 dilution, DAKO) for 30 minutes and then mouse APAAP (1:100 dilution, DAKO) for 30 mins after intervening washings with PBS. The slides were then developed in New Fuschin (DAKO), counter-stained with haematoxylin for 1-2 mins and finally dehydrated through graded alcohols and isopropanol before mounting in DPX.

CD 31 immunohistochemistry – DAB method

Tissue sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and placed into deionised water. Antigen retrieval was achieved by microwave heating in citrate buffer (100mls concentrated buffer in 900mls distilled water) for 3x5 mins. Sections were left for 20 min in hot buffer before cooling in water. Endogenous peroxidase activity was quenched with a 30 min incubation in 3% hydrogen peroxide in distilled water at room temperature. To reduce non-specific staining, sections were incubated in first avidin containing block and then biotin block for 10 mins each incubation with an intervening wash in PBS. After a further wash in PBS, slides were exposed to primary antibody at 4°C overnight (anti CD31 DAKO mouse anti-human, 1:20 dilution). Slides were washed three times in phosphate buffered saline (PBS) followed by a 30 min incubation with the secondary antibody at room temperature (DAKO rabbit anti-mouse, 1:300 dilution). For signal amplification, the avidin-biotin complex (ABC) method was used. After incubation with ABC horseradish peroxidase (HRP,DAKO) for 30 min at room temperature and two washes with PBS, visualisation was achieved using diaminobenzadine DAB kit (DAKO) with a 5 min exposure. A further wash in PBS was performed and then counter-staining with haematoxylin for 1-2 mins was performed. Slides were then washed with tap water and acid alcohol to remove excess haematoxylin then placed in ammonia water to allow a blue colour to develop. Finally slides were dehydrated through graded alcohols and xylene before mounting in DPX.

Microvessel counts

Following immunohistochemical staining of endothelial cells as described, microvessel counts were performed by two observers: a consultant surgeon with previous experience and the investigator, who underwent a period of training with an expert in the field. Counts were performed using a conference microscope.

Following scanning of the CD31 stained sections at low power (x40) microvessel counts were performed under high power (x250) using a Chalkley eyepiece graticule (Fox 1995). This graticule with 25 randomly allocated dots was placed in the microscope eyepiece. Vessels were counted only if they lay under the dots. In order to take account of the volume occupied by the vessels, those with more than one dot overlaying them were counted more than once. Counts were performed in three high power fields and the sum of these counts gave the microvessel count for that specimen. Investigators performing the counts were blinded to diagnosis or timing of sample. Statistical analysis was performed with the InStat and SPSS program version 9.0 using appropriate statistical tests for individual comparisons e.g. Mann Whitney.

Chalkley 25 point eyepiece graticule

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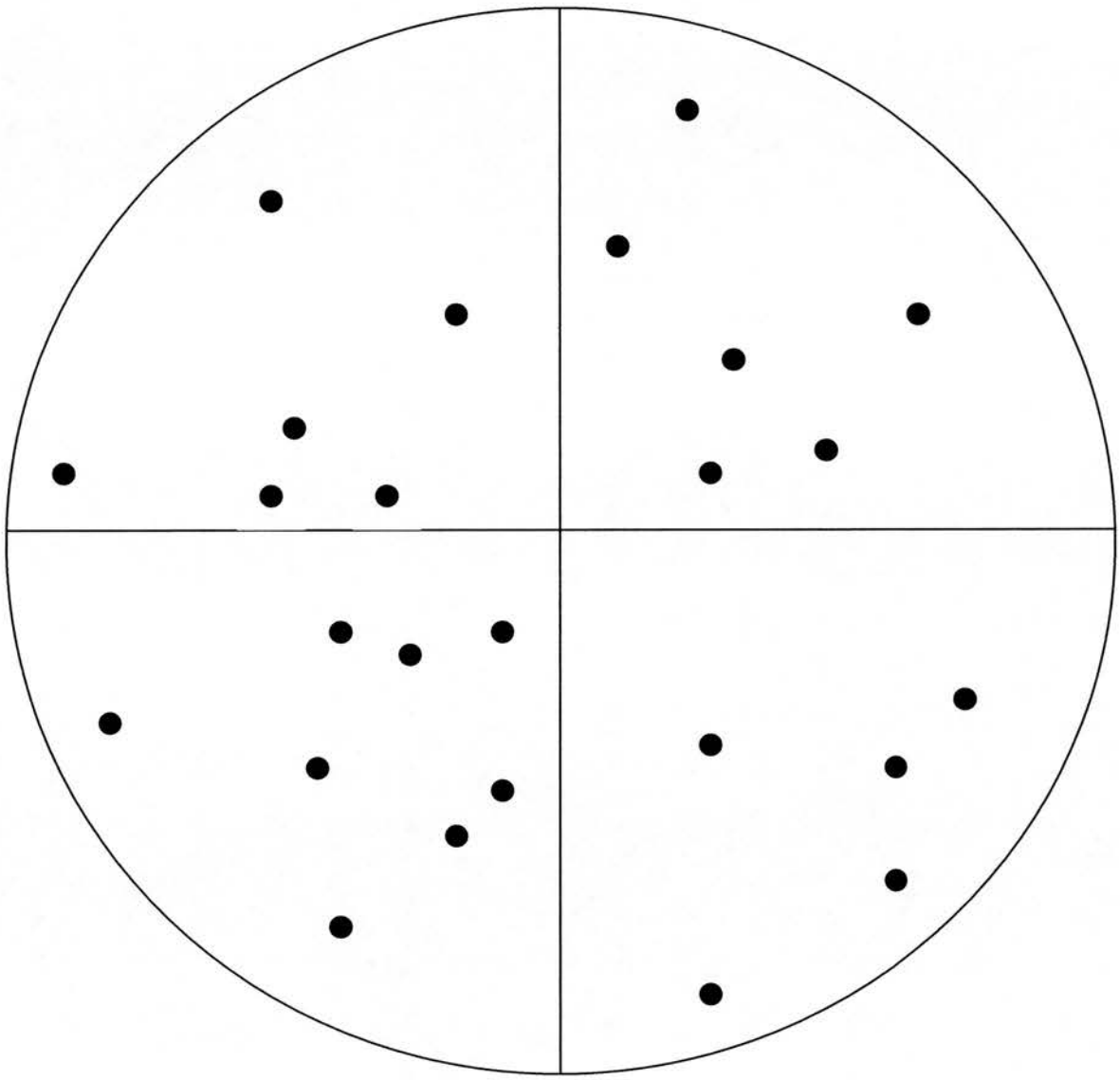


CD31/MIB1 dual immunohistochemistry

Staining for MIB1 was undertaken first and the method finished as per the protocol described above for the CD31 antibody from the stage of the primary antibody onward. Sections were dewaxed and rehydrated through graded alcohols to distilled water. Endogenous peroxidase activity was quenched with a 10 min incubation in 3% hydrogen peroxide in distilled water at room temperature. Antigen retrieval was achieved by microwave heating in citrate buffer (100mls concentrated buffer in 900mls distilled water) for 3x5 mins. Sections were left for 20 min in hot buffer before cooling in water. Incubation with avidin and biotin block for 10 min each was then undertaken with an intervening wash with TBS. DAKO protein block was applied for a 20 min incubation and the MIB-1 primary antibody (anti MIB-1 DAKO mouse anti-human, 1:150 dilution) applied immediately after for a 1 hour incubation at room temperature with no intervening wash. Secondary antibody was then applied (rabbit anti-mouse with biotin, 1:200 dilution) following washing with TBS and incubated for 30 mins. Slides were washed in TBS before incubation for 30 mins with the tertiary agent (Vector ABC Vectastain kit) with 3 drops applied per chamber. Washes with TBS and distilled water were followed by application of DAB to each slide for 5-10 min incubation until a brown colour developed. At this point, a further application of protein block was followed by the CD31 antibody as detailed above.

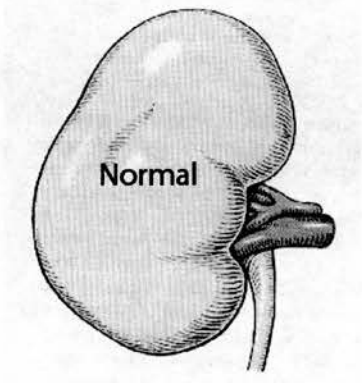
Proliferation scores

For each section dual stained for CD31 and MIB-1 absolute numbers of proliferating endothelial cells were counted and totalled from three fields seen under high power light microscopy (x250). A proliferation index (PI) was calculated for each specimen and was expressed as;

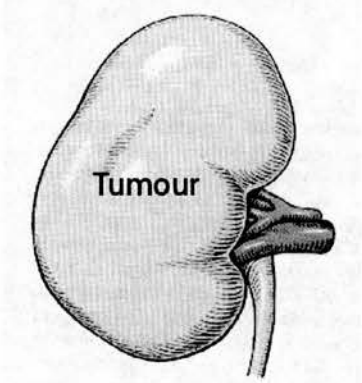
PI = Total no. of proliferating endothelial cells (staining pink and brown) / Total no. of proliferating cells (staining only brown) in same field.

Key to figures used in descriptions of study designs

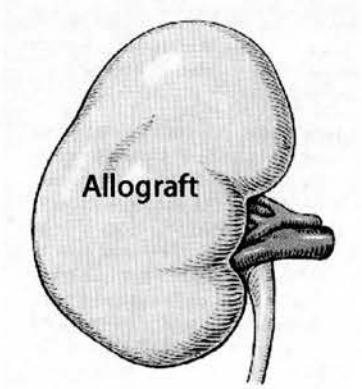
Normal kidney



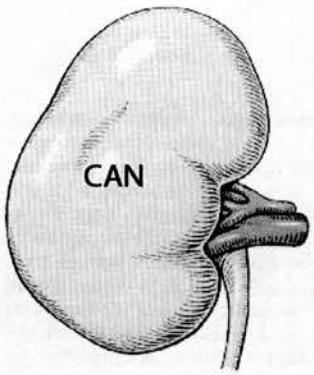
Renal tumour



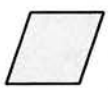
Renal allograft after transplantation



CAN nephrectomy specimen after excision



Cross section tissue specimen



Core biopsy tissue specimen

1) Assessment of Vascularity in Human Parathyroid Tissue

Patient material

Having obtained ethical approval and patient consent, parathyroid tissue was obtained from patients undergoing elective parathyroidectomy as treatment for hyperparathyroidism either due to a parathyroid adenoma or in the setting of chronic renal failure with four gland hyperplasia. Tissue was taken for frozen section analysis to confirm it to be parathyroid in origin and remaining tissue made available for study. For histopathological evaluation and in situ hybridisation, parathyroid tissues were immediately fixed with 4% paraformaldehyde and 5μm paraffin sections were cut. For Western blot analysis, parathyroid tissue was stored at -70˚ until required for use and approximately 1 mm^3 needed to extract protein lysate. Fresh tissue was obtained from one normal gland, three separate adenomas and from three patients with four gland hyperplasia.

Following ethical approval, formalin fixed, paraffin embedded histology blocks were also available from patients who had undergone parathyroidectomy over the preceding 11 years and these specimens were cut from archive specimens kept following diagnosis. Four or five sections were obtained from each of 123 specimens.

Study design

To determine differences in vascularity between normal human parathyroid tissue, parathyroid hyperplasia and adenoma, histological sections from 123 patients were obtained in this retrospective study. Following immunohistochemical staining with antibody to CD31, microvessel counts were performed on 44 normal, 27 hyperplastic and 52 adenomatous glands.

In addition, 91 of these sections (36 normal, 10 hyperplasia and 45 adenoma) were immunostained with antibody to VEGF to assess for its expression and a further 15 sections (5 of each type) underwent in situ hybridisation to assess for levels of VEGF mRNA. Protein was extracted from 5 specimens of parathyroid tissue (1 normal, 2 hyperplasia and 2 adenoma) and Western blotting analysis performed with antibody to VEGF.

A correlation between changes in vascularity and expression of VEGF protein and mRNA was sought following these analyses.

VEGF immunohistochemistry

Tissue sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and placed into deionised water. Endogenous peroxidase activity was quenched with a 5 min incubation in 3% hydrogen peroxide in distilled water at room temperature. Antigen retrieval was achieved by microwave heating in citrate buffer (100mls concentrated buffer in 900mls distilled water) for 3x5 mins. Sections were left for 20 min in hot buffer before cooling in water and then washing with TBS. Incubation with avidin and biotin block for 10 min each was undertaken with a wash with TBS after each. A serum free protein block was applied for 5-10 mins and not washed off prior to incubation for 2 hours at room temperature with the primary anti-VEGF antibody (rabbit anti-human VEGF polyclonal,1:400 dilution, sc-152 Santa Cruz Biotechnology, Santa Cruz, CA). Negative control slides were exposed to primary antibody and VEGF blocking peptide (1:80 dilution, sc-152p Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody was washed off with TBS and secondary antibody applied (goat anti-rabbit, 1:300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for a 30 min incubation. Slides were then washed with TBS. For signal amplification, the avidin-biotin complex (ABC) method was used. After incubation with ABC horseradish peroxidase (HRP, DAKO) for 30 min at room temperature and two washes with PBS visualisation was achieved using DAB kit (DAKO) with a 5 min exposure. Sections were washed in PBS and then counter-stained with haematoxylin for 1-2 mins. Slides were washed with tap water and acid alcohol to remove excess haematoxylin and placed in ammonia water to allow a blue colour to develop. Finally slides were dehydrated through graded alcohols and xylene before mounting in DPX. Positive control tissue from human endometrium was used.

VEGF assessment

Sections of parathyroid tissue stained with antibody to VEGF were scored by two observers according to overall proportion and intensity of staining as compared to the positive control of normal human endometrium. Assesments were made in three high power fields (x250) and the total of three scores was documented. Intensity of staining was compared to the positive control for each staining run. The semi-quantitative scoring system used was as follows:

Intensity; 0 – no staining

1 – traces of staining/mild staining

2 – moderate staining

3 – dark/strong staining

Proportion; 1 – 0 to 25% of all cells staining

2 – 25 to 50% cells staining

3 – 50 to 75 % staining

4 – 75 to 100% staining

In situ hybridisation to VEGF

In situ hybridisation was performed using cRNA probe for human VEGF. For each slide a sense and anti-sense was carried out. Sense and anti-sense probes were prepared using an RNA transcription kit (Ambion, Austin, TX) and labelled with 35S-uridine 5'-triphophate (NEN, Boston, MA). Tissue is fixed in 4% neutral buffered formalin for 24 hours. Serial paraffin sections 5 microns thick were cut and mounted onto BDH Super Frost Plus (cat. No. 406/0179/00) coated glass slides. The sections were rehydrated through xylene bath followed by graded alcohols, 0.2N HCl and then finally depc (0.1% diethyl pyrocarbonate) water. The sections were treated with proteinase K (5μg/ml, Sigma, St. Louis, MO) for 30 min at 37°C then taken through 0.2% glycine for 10 min at 4°C, triethanolamine buffer (TEA) for 5 min, TEA buffer with acetic acid for 10 min and then SSC (NaCl FW 58.44, Sigma S-3014 with tri-sodium salt FW294.1, Sigma C-8532 made to pH 7.0 with HCl in pure water) for 5 min. 100μl of pre-hybridisation buffer was added to each slide and incubated at 55°C for 2 hours. Just before use the probe was denatured at 95°C for 10 min then put on ice. Once pre-hybridisation was complete the excess buffer was dried off and 50μl of hybridisation buffer was added to each. Subsequent hybridisation was performed in a moist chamber overnight at 55°C. High stringency post-hybridisation washings and RNAase treatment were used to remove excess probe. Slides were dehydrated and air dried for 4 hours prior to dipping in emulsion (Ilford G5 liquid emulsion, H.A.West, Edinburgh, UK). Slides were then left in a light proof humid box with exposure time of 7 weeks. Subsequent development of the slides took place with Kodak D19 developer (Kodak, Rochester, NY) at 4°C and fixed with Kodak GBS fixer Kodak, Rochester, NY) then counterstained with haematoxylin (Richard-Allan, Richland, MI), dehydrated and mounted.

In situ hybridisation to VEGF was evidenced by the presence of clustering of silver grains within cells seen under dark field microscopy in the anti-sense sections and compared against a positive control of human corpus luteum and negative control of corresponding sense sections.

Western blot analysis to VEGF

Protein extraction: Parathyroid tissue obtained from patients undergoing elective parathyroidectomy was stored at -70˚C until required. Tissue was then removed but kept frozen in liquid nitrogen (BOC, UK) and samples no greater than 5mm in diameter were freeze fractured using a Bio-Pulverizer Mini Dismembranator (Braun, Germany). The resultant powder was removed and added to the protease and cell lysis buffer and vortexed. The supernatant was removed and a protein assay performed using the Bio-Rad protein assay kit. Final concentrations were obtained for each tissue to allow calculation of the volume of suspended lysate required to perform the Western blot analysis.

Western (immuno) blotting protocol; Following denaturing at 90˚C for 5 mins (Tweedle Dum) equal amounts of the lysate proteins (20μg) were loaded onto a 12.5% SDS polyacrylamide gel and separated with a 200 volt potential difference for approximately one hour. In addition to the test samples, two additional lanes were run, one containing known molecular weight markers for the reference range of interest for VEGF and the other being a purified VEGF protein product (Santa-Cruz sc-152p, Santa-Cruz Biotechnology,Ca). The proteins were then transferred to a nitrocellulose membrane at 80mA for one hour ('Trans-Blot' transfer medium, 0.45μm, Bio-Rad, Hercules, CA)). The nitrocellulose was soaked in Ponceau red solution (0.1% Ponceau S, 5% acetic acid, Sigma, St Louis, MO) to allow visualisation of protein standards and lanes which were marked on the membrane in pencil. Excess Ponceau red was washed off with TBS several times before the blocking agent is applied. A 5% TBS/skim milk powder (Marvel) solution was added to the membrane which was blocked for one hour at room temperature. The membrane was then removed from the blocking solution and without an intervening wash the primary antibody was added. The primary antibody to VEGF (rabbit anti-human, 1:1000 dilution, Santa-Cruz sc-152, Santa-Cruz Biotechnology, Ca) was diluted in 1% bovine serum albumin (Sigma, St Louis, MO) and Marvel solution of TBS, 0.05% Tween and incubated for one hour at room temperature. Several washes

with TBS Tween 0.05% were performed before the secondary antibody (HRP-conjugated goat anti-rabbit, 1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) applied for a 40 min incubation at room temperature. Two further washes with TBS Tween 0.05% were performed followed by a final wash in TBS before the membrane was ready for development. The signals were detected by ECL Western blot analysis system (ECL WB Detection Reagents, Amersham Biosciences, Bucks, UK).

Presence of VEGF protein was detected by the presence of a protein band at the correct molecular weight as compared to the control VEGF protein product and the protein standards.

2i) Validation of methodology – observer variation

Assessment of angiogenesis in histological sections commonly involves the use of microvessel counts or microvessel density (Ozdemir et al 2001, Oda 2003, Kasper et al 1999, Marson et al 2001). Tissue heterogeneity and observer variation make this methodology susceptible to problems with reproducibility. To improve objectivity of microvessel counts the use of a Chalkley eyepiece graticule or computerised image analysis have been advocated (Fox et al 1995). This has been shown to generate reproducible results thus minimising intra- and inter-observer variation (Marson et al 1999) which have previously been reported (Axelsson et al 1995).

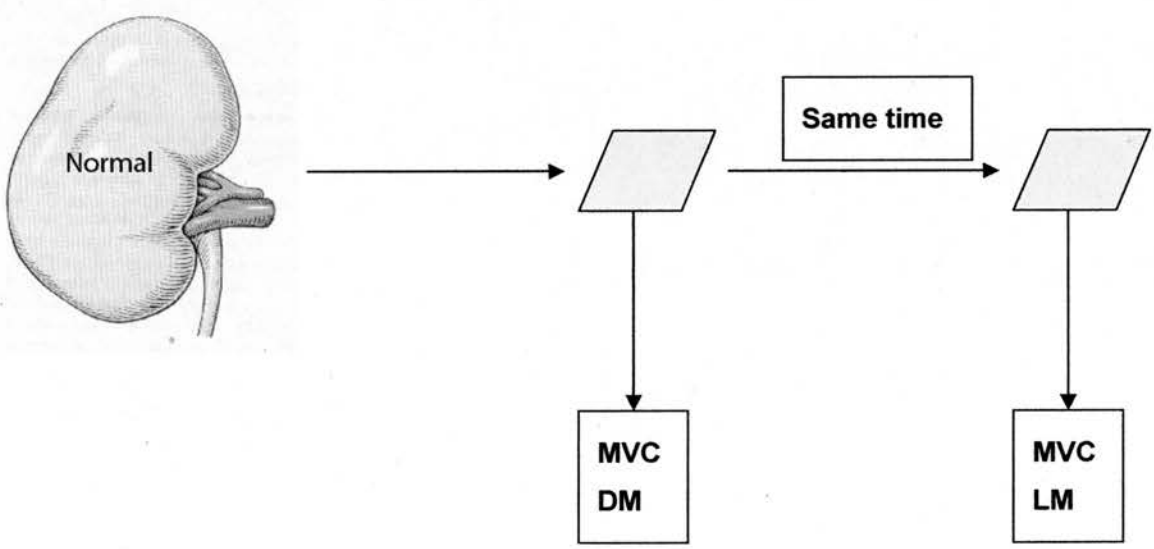
Patient material

For determination of intra-observer variation and inter-observer variation specimens collected from other aspects of the study were studied. Normal kidney came from those collected as described or from additional sections of normal kidney retrieved from archive material following ethical approval and in collaboration with the pathology department.

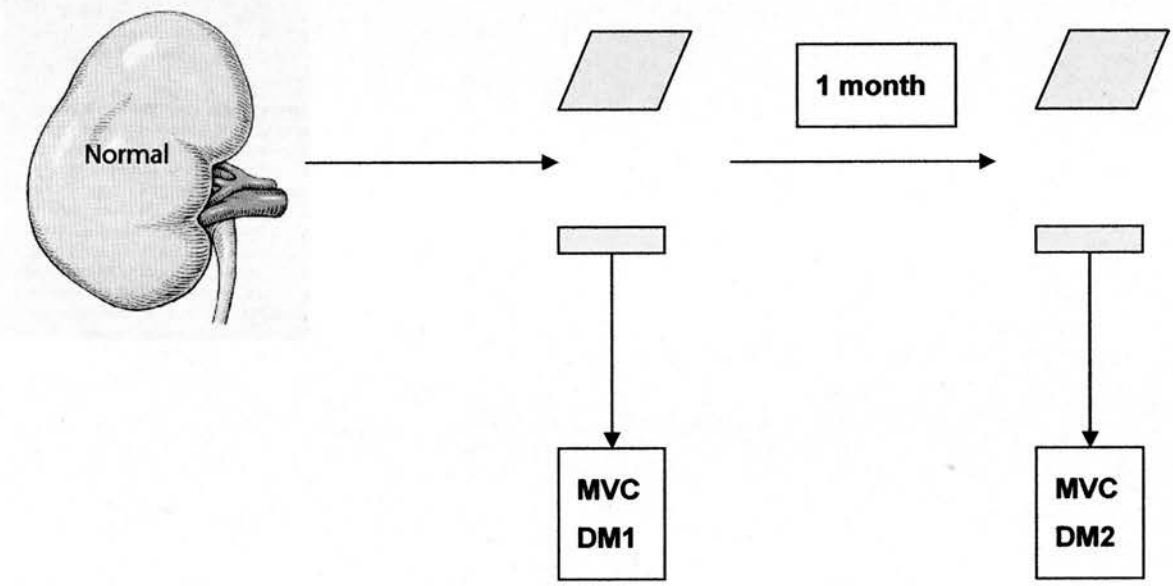
Study design

Intra- and inter observer variation were assessed. Microvessel counts were obtained following staining of normal kidney sections with antibody to CD31. These counts were performed on forty-five sections by observer DM at different time points and separately by two observers DM and LM on 25 sections.

Comparison of inter-observer variation between investigators DM and LM



Comparison of intra-observer variation between two time points for investigator DM



2ii) Validation of methodology – reproducibility study

There is recent evidence to suggest that microvascular changes contribute to certain progressive renal diseases and that further understanding of the process of angiogenesis may lead to novel therapeutic strategies in their treatment (Kang et al 2002, Kang et al 2003). Angiogenesis has been shown to be implicated in the development of renal cell carcinoma (Oda et al 2003, Le Jan et al 2003, Dekel et al 2002), differing forms of glomerulonephritis (Honkanen et al, Masuda et al 2001, Noguchi et al), glomerulosclerosis (Kang et al 2002) and renal transplant nephropathy (Pilmore et al 1999, Ozdemir et al 2001). The use of renal biopsy tissue in the study of renal disease provides a useful source of material for research particularly in the study of chronic allograft nephropathy where serial specimens are often available (Bellamy et al 2000, Freese et al 2001, Legendre et al 1998, Pilmore et al 1999).

It is well documented that heterogeneity of vasculature is exhibited in certain human tumours and that microvascular 'hot spots' are seen e.g. breast cancer (Weidner et al 1991, Van Hoef et al 1993). Care must be taken when interpreting microvessel counts derived from core biopsies as they may not accurately represent the whole tissue under study which will limit their use as a means of assessing the microvasculature (Jacobs et al 1998). These concerns regarding accuracy of performing microvessel counts on core biopsy specimens should not be limited solely to the study of tumours but also to other normal and diseased tissues, although it is often assumed that there exists homogeneity of vasculature in normal tissue.

Patient material

Having obtained regional ethical approval and written patient consent tissue was obtained from 27 patients undergoing elective nephrectomy, 25 for renal tumours, 1 for obstructing ureteric tumour and 1 for chronic pyelonephritis. Non-tumour tissue was defined as 'normal' kidney and this was available from all but one of the specimens (a partial nephrectomy where only tumour tissue was removed). Following removal of the kidney the specimen was immediately assessed by the pathologist and between one and three random biopsies of the normal kidney and the tumour (where present) were performed with a disposable 14 gauge Tru-Cut biopsy needle. Cross section specimens were then cut by the pathologist from the same area to allow comparison with biopsies from the same kidney.

As a separate study, paired samples of tumour cross section and biopsy were also performed, thus each specimen provided two pairs of samples (normal biopsy and cross section and tumour biopsy and cross section per kidney).

All specimens were fixed in formalin for at least 24 hours and paraffin-embedded following usual tissue processing through heated alcohols, xylene and wax. Sections of 4μm were cut from each specimen to allow for immunohistochemical assessment.

Study design

To compare results obtained from core biopsy and cross section specimens taken from the same kidney at the same time, immunohistochemical staining was performed with antibody to CD31.

In 27 excised kidneys, 26 core biopsy and cross section of normal kidney obtained at the same time were available for analysis.

• Direct comparison of microvessel counts between normal core biopsies and the corresponding normal kidney sections was undertaken. Separate microvessel counts were taken from the cortex and medulla from each section (Figure a).

• Similar direct comparison of microvessel counts between tumour biopsies and corresponding tumour cross section was undertaken (Figure b).

• Additionally direct comparison of microvessel counts between cross section of normal kidney and tumour from the same kidney was undertaken (Figure c).

Figure a - Comparison of MVC between cross section and core biopsy from same kidney at same time point (Figure a

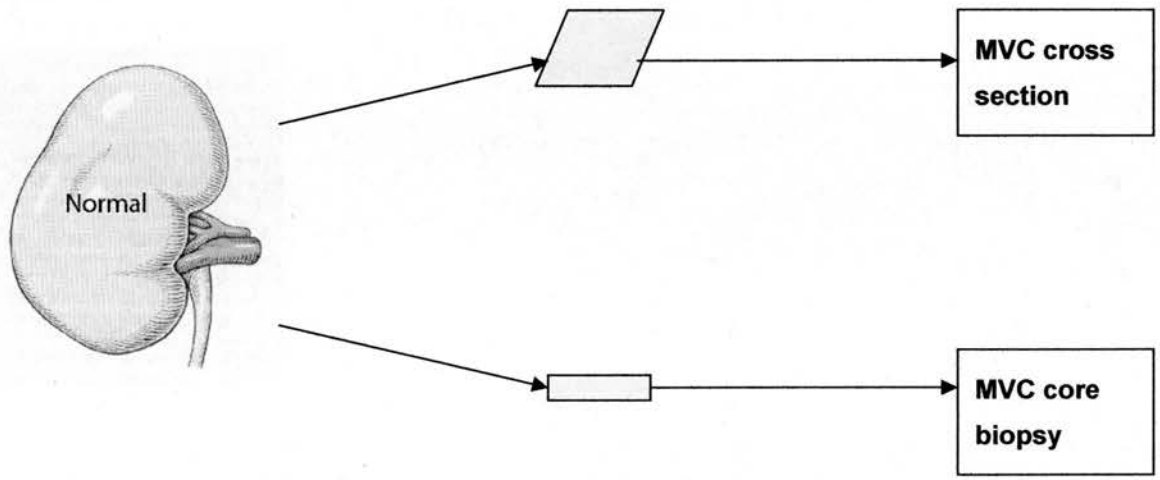


Figure b - Comparison of MVC between cross section and core biopsy from same kidney tumour at same time point

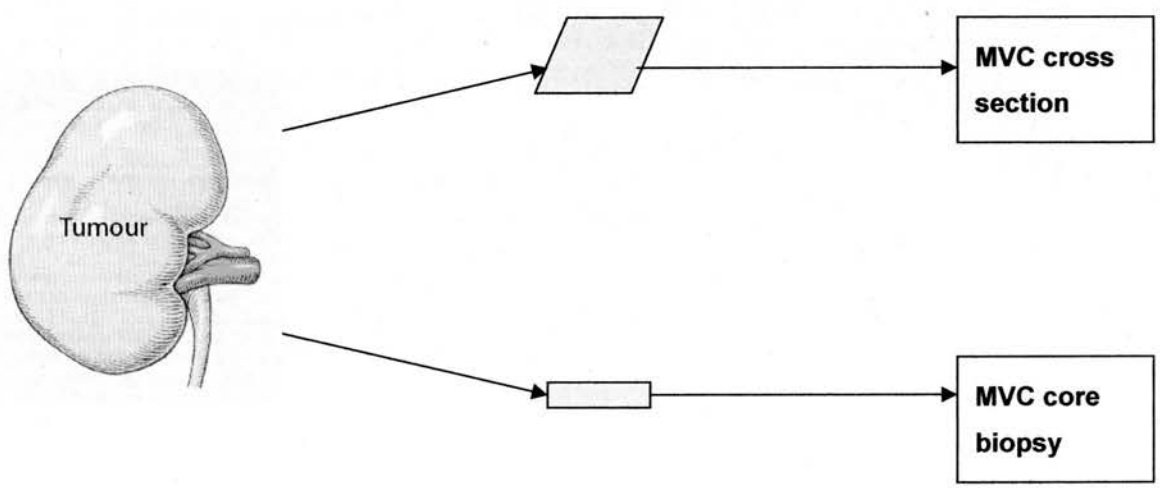
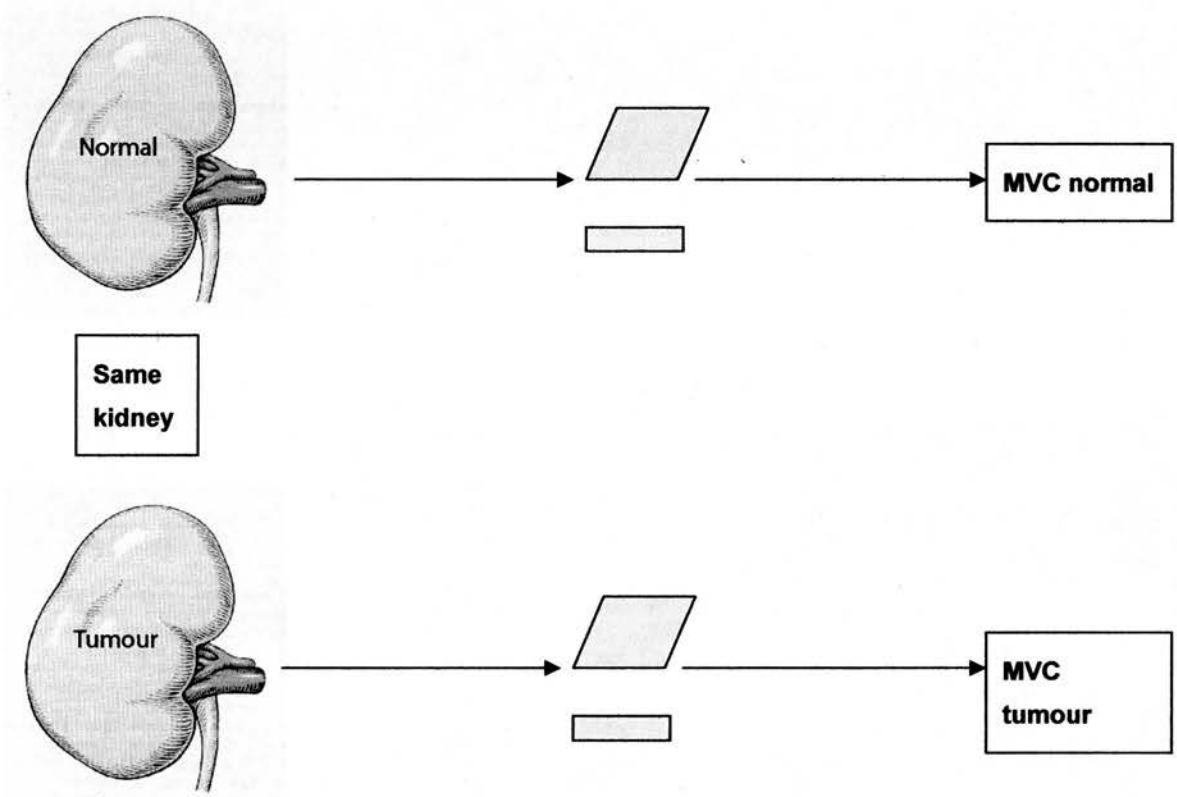


Figure c - Comparison of MVC between normal kidney and kidney tumour (cross section and core biopsy) at same time point



3) Changes in vascularity, endothelial cell proliferation and macrophage infiltration in renal allograft nephrectomies with CAN compared with normal kidney

This study compares results of normal kidney with renal allograft nephrectomy specimens performed because of chronic allograft nephropathy. This group of patients represents end stage CAN when graft function has failed, immunosuppressive therapy ceased and renal replacement therapy recommenced.

Patient material

Having obtained regional ethical approval, all pathology reports from transplant nephrectomies performed at the Royal Infirmary of Edinburgh between 1991 and 2002 were reviewed by a pathologist and those which showed changes of chronic allograft nephropathy were selected for study. A total of 34 nephrectomies from 31 different patients were identified (3 patients had 2 grafts studied). Median graft duration was 49 months (range 2 -189) with median age of recipient 45 years at time of nephrectomy (range 30 – 69). There were 18 male and 13 female recipients from 1 living-related and 33 cadaveric donors.

Normal kidney from 62 patients was available with median age of patient 53 years at time of nephrectomy (range 31 - 80). There were 37 male and 25 female patients.

Study design

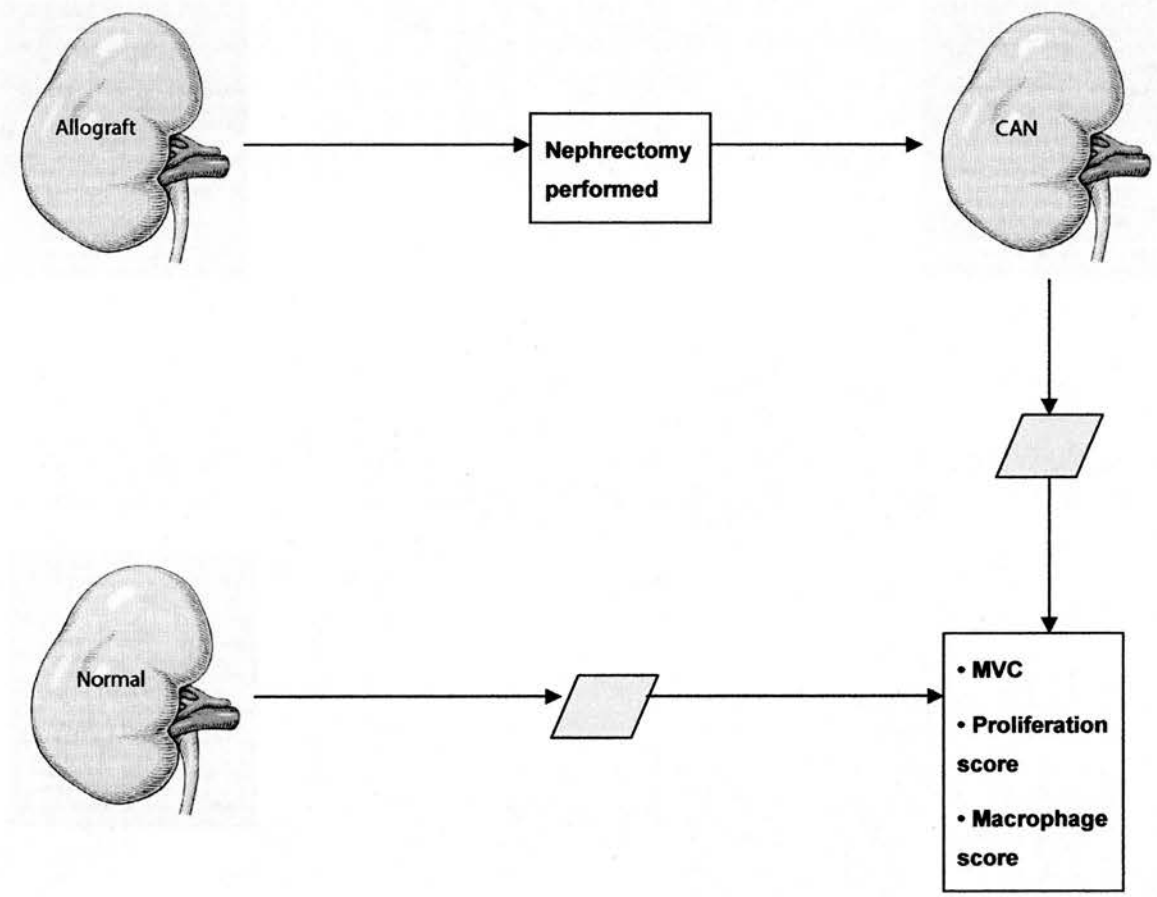
Immunohistochemical analyses were performed to compare normal kidney with the CAN nephrectomy specimens which were:

- CD31 staining and microvessel counts

- CD31/MIB-1 dual staining and assessment of endothelial cell proliferation and proliferation index

- CD68 staining and glomerular macrophage counts

Comparison between normal kidney and allografts performed because of CAN



4) Sequential changes in vascularity, endothelial cell proliferation and macrophage infiltration during CAN using renal allograft biopsies

This study was undertaken in two parts:

- early allograft biopsy vs. endstage CAN specimen (paired samples)

- protocol allograft biopsies comparing those grafts that went on to develop CAN with those with continued stable function.

i) Renal allograft biopsy specimens obtained because of graft dysfunction in the cohort of patients studied who progress to nephrectomy due to chronic allograft nephropathy were analysed. This group of biopsy specimens were taken at varying time points after transplantation but before graft failure and nephrectomy. The specimens were compared with the cross section from the nephrectomy for each patient and with normal kidney. This allows investigation of changes that occur during the progression of CAN.

ii) Protocol biopsies taken early after transplantation were studied. This population of patients contained grafts which went on to develop CAN and those in which CAN did not develop and stable graft function ensued. This allowed for investigation as to whether grafts which develop CAN show early changes which may have prognostic or diagnostic importance.

Patient material

31 transplant biopsies performed to assess the cause of deterioration in renal function were obtained from 22 of the 34 grafts that went on to develop CAN. These are referred to as 'clinical biopsies' in the remainder of the thesis. Biopsies were taken at differing time points during the life of each graft (median biopsy at 17 months after transplantation, range 1 week to 96 months). All diagnoses from these biopsies were included for study. These were; 4 normal, 9 acute rejection, 11 CAN, 7 other.

Additionally, 28 sections from protocol biopsies were studied and MVCs obtained. Stable graft function developed in 20 patients (median time of biopsy 20.5 days after transplantation) and 8 developed CAN with associated deterioration in graft function (median biopsy at 19 days).

Study design

Three different immunohistochemical stains were applied to the clinical biopsy group and this allowed for comparison with CAN nephrectomy tissue and normal kidney.

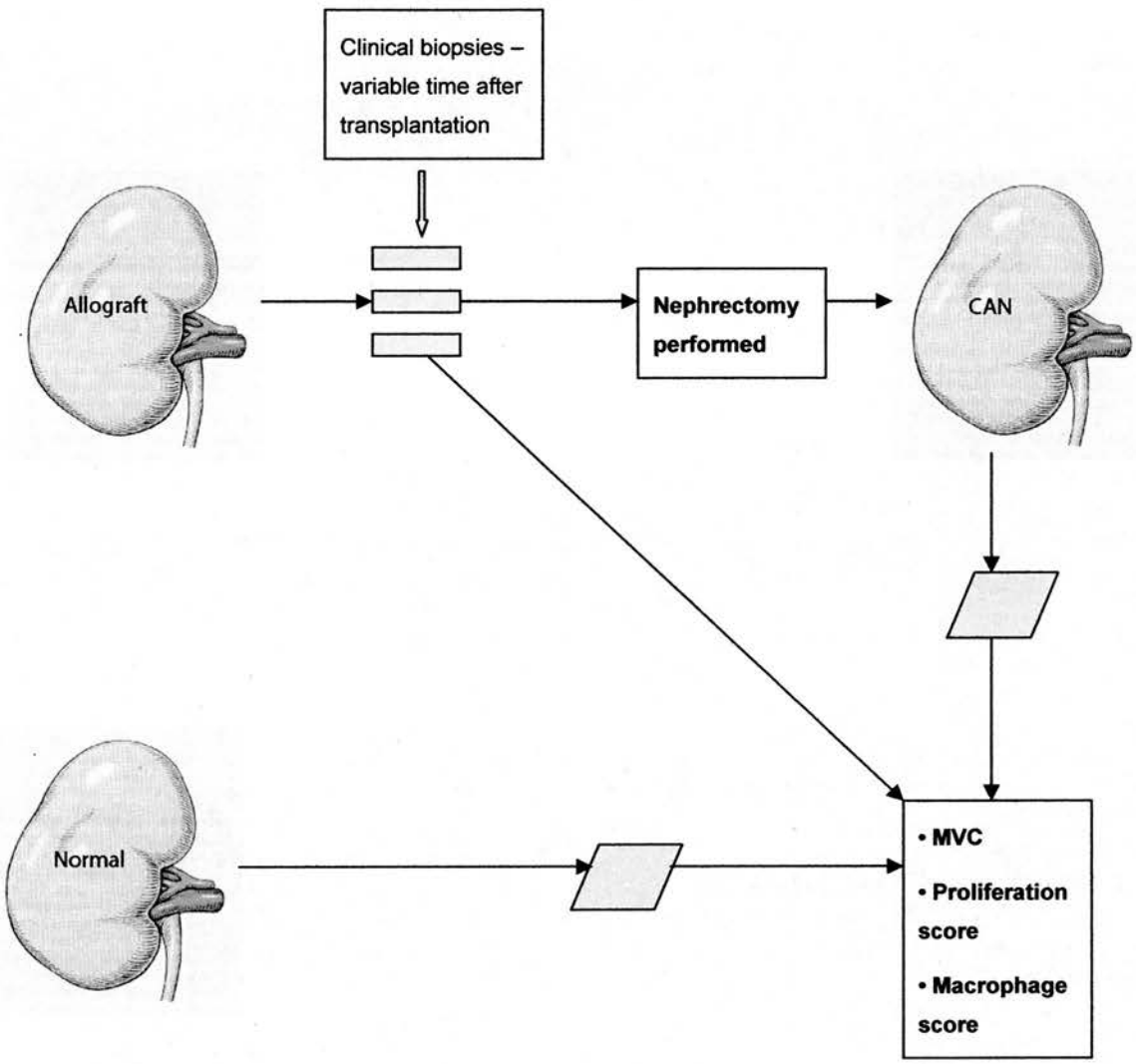
- CD31 staining and microvessel counts

- CD31/MIB-1 dual staining and assessment of endothelial cell proliferation and proliferation index

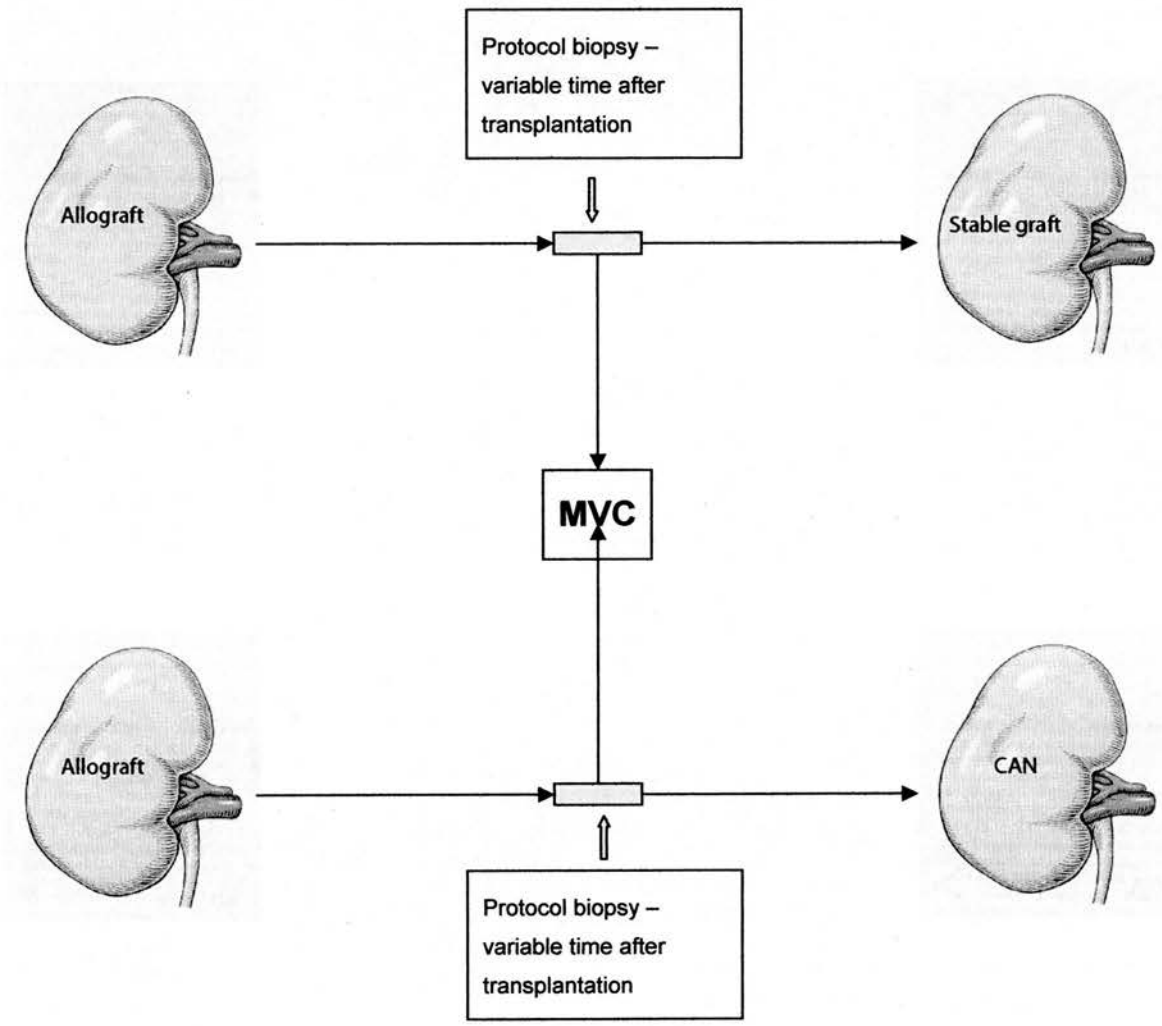
- CD68 staining and glomerular macrophage counts

Protocol biopsies were only immunostained with antibody to CD31 and microvessel counts performed.

Comparison of clinical biopsies with corresponding CAN nephrectomy and normal kidney



Comparison of protocol biopsies that develop CAN with those that enjoyed stable graft function



Chapter 3 Parathyroid Results and Discussion

This section will detail all results obtained during experiments with parathyroid tissue. Tables and graphs are presented for:

- significant positive results obtained with CD31 staining and microvessel counts

as well as negative results obtained from:

- VEGF immunohistochemistry,
- MIB-1/CD31 dual staining immunohistochemistry,
- VEGF in situ hybridisation and
- VEGF Western blotting

A discussion of these results then follows in this chapter with the remainder of the thesis focussing on studies of chronic allograft nephropathy.

Parathyroid results

Microvessel counts

123 patients were studied (44 normal, 27 hyperplasia and 52 adenoma). Microvessel counts were as follows:

Table 3.1: Microvessel counts

	Normal	Hyperplasia	Adenoma
Mean	13.64	24.25	31.27
Standard deviation (SD)	7.64	8.94	10.67
Standard error of mean (SEM)	1.311	1.690	1.609
Median	13.5	26	34
Range	2 -43	8 -56	10-54

There was a statistically significant and step-wise increase in microvessel count between the different types of tissue (Mann Whitney cmparison of means).

Normal vs Hyperplasia: $p<0.001$

Normal vs Adenoma: $p<0.001$

Hyperplasia vs Adenoma: $p=0.0027$

Figure 3.1: Comparison of microvessel counts in normal, hyperplastic and adenomatous parathyroid glands.

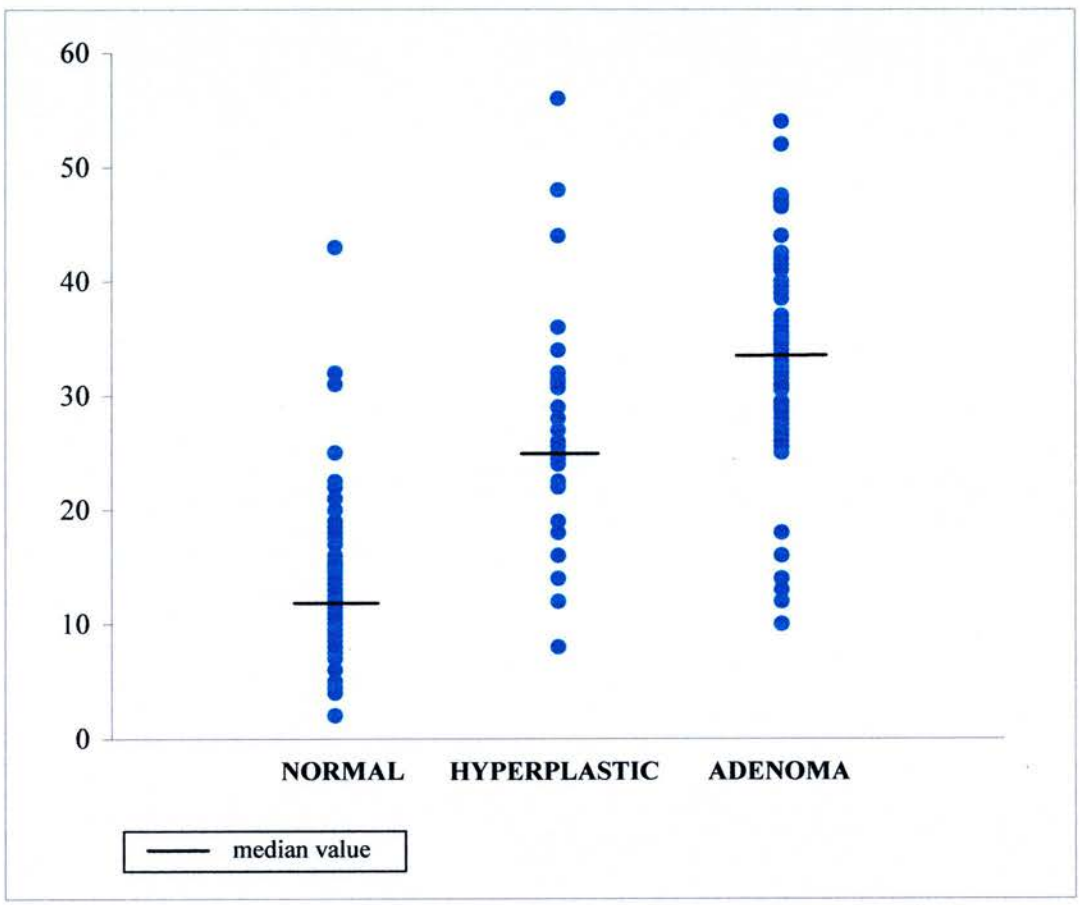


Figure 3.2 gives examples of CD31 staining of normal parathyroid, hyperplasia and adenoma with microvessel appearing brown.

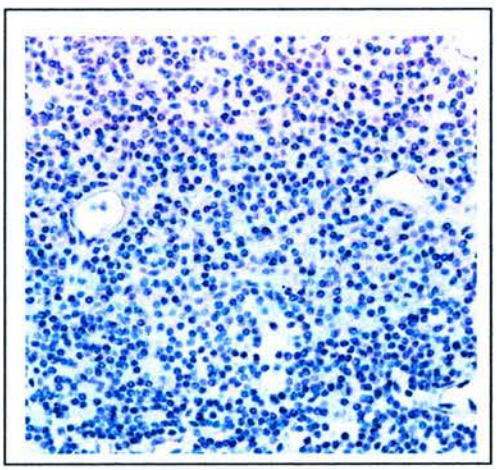


Figure 3.2a. Normal parathyroid; Less CD31 staining (x20) evident.

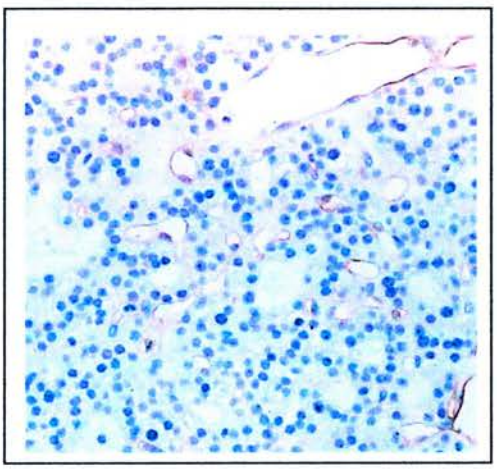


Figure 3.2b. Parathyroid hyperplasia; Prominent CD31 staining (x20).

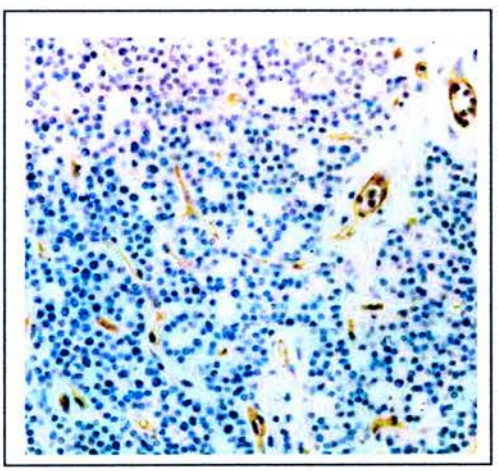


Figure 3.2c. Parathyroid adenoma; brown CD31 staining (x20) apparent throughout.

Immunohistochemistry

91 sections of parathyroid tissue (36 normal, 45 adenoma and 10 hyperplasia) were immunostained with antibody to VEGF. Intensity and proportion of cells staining were as follows:

Table 3.2: VEGF IHC scores

	Intensity				Proportion			
	0	1	2	3	1	2	3	4
Normal	4	14	14	4	26	7	3	0
Adenoma	9	20	11	5	36	6	3	0
Hyperplasia	4	2	4	0	8	2	0	0

Table 3.3: VEGF scores – mean, median, SEM and range

	Mean total score	Median score	Std. error of mean (SEM)	Range
Normal	3.18	3	0.1991	0 -7
Adenoma	2.39	2	0.2015	0 - 4
Hyperplasia	2.69	2	0.2627	0 – 4

Statistical analyses comparing mean total scores is shown below (Mann Whitney):

Normal vs Hyperplasia: $p = 0.2321$

Normal vs Adenoma: $p = 0.0316$

Hyperplasia vs Adenoma: $p = 0.6619$.

58% of all sections showed either zero or mild staining (53 of 91 sections) and thus that 42% of sections show either moderate or strong staining. Furthermore, the degree of intensity of staining showed a similar distribution between the differing types of tissue and there is no step-wise increase in intensity of staining between normal, hyperplasia and adenoma as was seen with the microvessel counts.

These data also confirm that in 77% of sections (70 of 91), the proportion of cells staining for VEGF at any intensity level is less than 25% of cells on each section. Only 6.6% (6 of 91) of sections showed a similar proportion of cells staining (>50% or groups 3 and 4 in the table above) to those of the positive control sections. Again these data show no difference in the distribution of the proportion of cells staining for VEGF between the different types of tissue.

However, taking the mean total score obtained does reveal a statistically significant difference between normal tissue and adenoma ($p=0.0316$).

Figure 3.3: Comparison of VEGF scores in normal, hyperplastic and adenomatous glands.

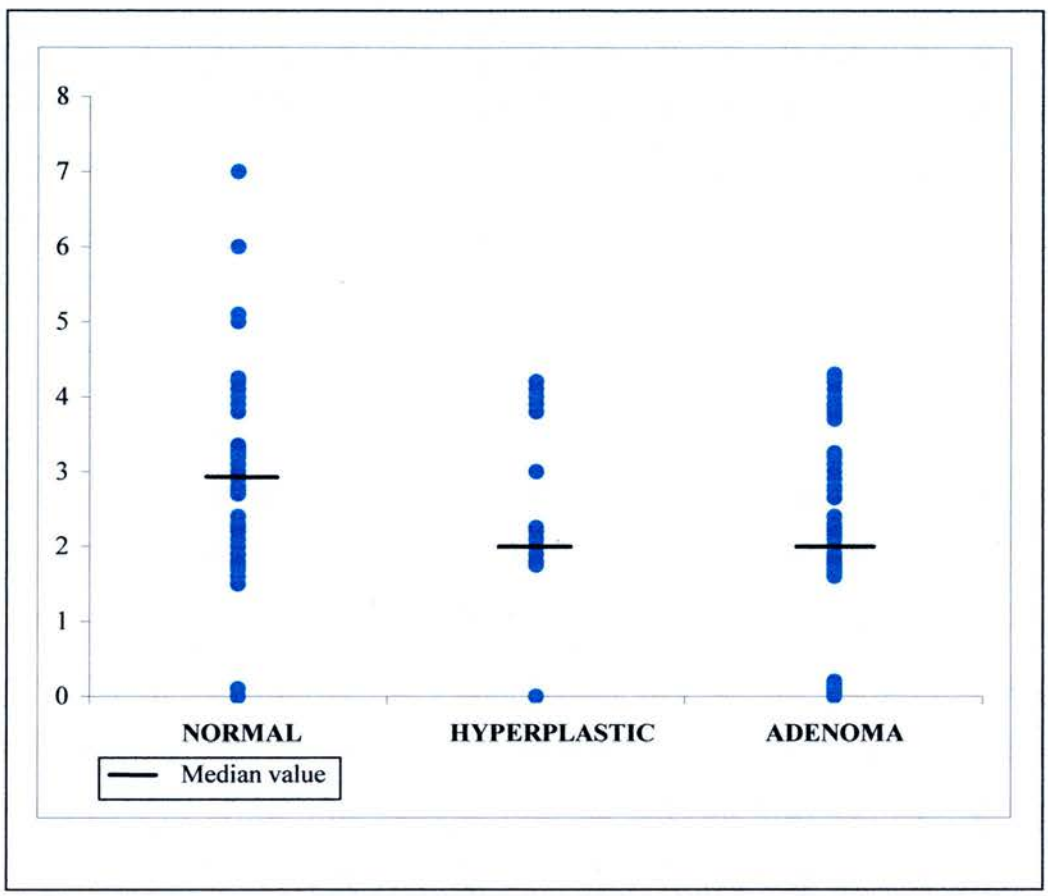


Figure 3.4: Examples of IHC with antibody to VEGF

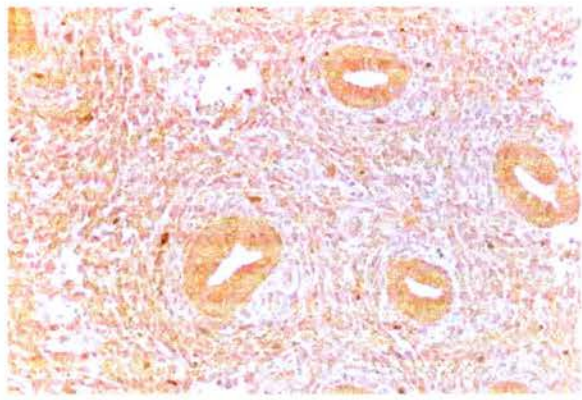


Figure 3.4a.Positive control VEGF IHC (immunohistochemistry) (x20), Human endometrium; Strongly staining brown with specificity compared to negative controls.

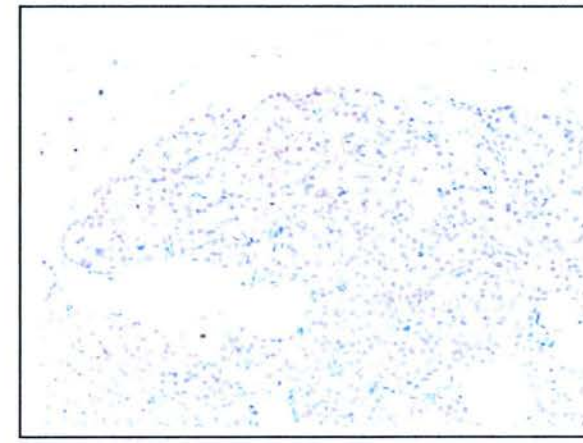


Figure 3.4b.Parathyroid adenoma VEGF IHC (x20) showing less intense and less frequent staining pattern than positive control.

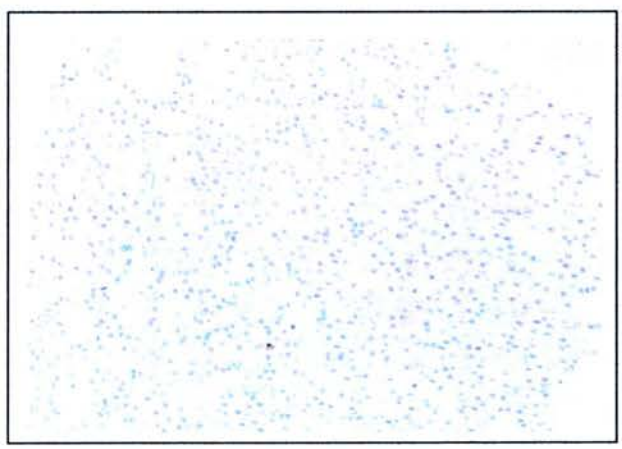


Figure 3.4c.Parathyroid hyperplasia VEGF IHC (x20) showing similar staining to adenoma and normal tissue.

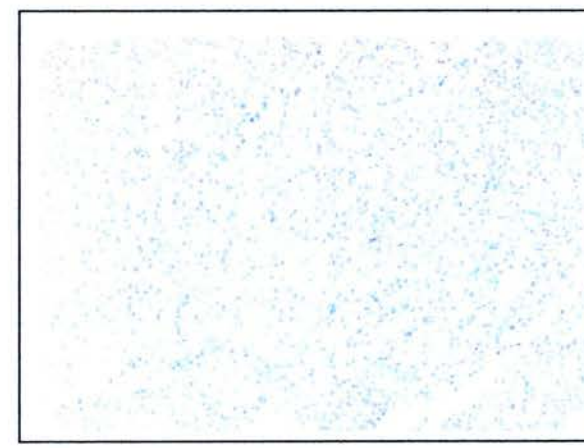


Figure 3.4d.Normal parathyroid VEGF IHC (x20) showing staining similar to adenoma and hyperplasia.

CD31/MIB-1 Dual Stain

133 sections were dual stained with antibodies to CD31 and MIB-1 (which stains the nuclear protein Ki-67, a marker of cells undergoing proliferation) as described above. Endothelial cells were visualised under a light microscope by a characteristic reddish pink colour as determined by the staining method used to detect CD31. MIB-1 positive cells were identified by a characteristic dark brown staining of the nucleus. Proliferating endothelial cells were thus identified as those with both red and brown staining. Three high power fields (x250) were selected at random and cell counts performed for the total number of proliferating cells per field as well as the total number of proliferating endothelial cells. After analysis of 57 sections:

- the maximum number of proliferating endothelial cells in any given field was 1

- these cell types were only seen in 14% (8 of 57) of the sections studied

- 29 sections (51%) showed no proliferating cells of any kind.

- proliferating endothelial cells were visualised in both normal and adenoma sections (but not hyperplasia) but not in significantly differing numbers.

Scanning of the remaining 77 sections demonstrated a similarly low number of proliferating cells and formal counting was not performed. These findings do not offer an explanation to the significant increase in microvessel counts seen in hyperplasia and adenoma as detailed above. It might have been expected that the increase in microvessel count was associated with a significant increase in the number of proliferating endothelial cells.

Consultation with a senior consultant pathologist revealed that owing to the morphology of small blood vessels and the thickness of sections cut that identification of proliferating endothelial cells would probably require analysis of serial sections from each patient. With the exception of parathyroid samples

collected prospectively form the start of the study such sections were not available for analysis. This therefore is an area of further study.

In Situ Hybridisation

Sense and anti-sense sections of parathyroid tissue from 5 normal, 5 hyperplasia and 5 adenoma were available for study. Exposure occurred at 11 weeks with trial sections developed at the 3 and 6 week stage. Sections were counterstained with haematoxylin and viewed under light and dark field microscopy to identify clustering as areas of positive expression.

	Corpus luteum	Normal parathyroid	Hyperplasia	Adenoma
Clustering present – antisense	Yes	No	No	No
Clustering present – sense	No	No	No	No

Clustering was absent in all sense sections studied as expected for negative control. Clustering was present in all antisense sections of positive control corpus luteum developed after a 6 week exposure as per normal protocol for this tissue. Tests slides of each type of parathyroid tissue were studied at 3 and 6 weeks and no clustering was present. The study sections were left for an 11 week exposure prior to development. Again these sections showed no evidence of hybridisation to VEGF in any type of parathyroid tissue.

Western blotting analysis

The protein lysate from parathyroid tissue form 1 normal gland, 3 adeonomas and 4 hyperplastic glands (from the same patient) were studied. The presence or absence of a band of protein at the same latitude as the positive control purified VEGF product (approx. 46 kDa) was used as the criterion for whether each type of tissue expressed VEGF in vivo at the time of specimen collection. Results with first antibody trialled (Santa-Cruz polyclonal rabbit anti-human) showed that no tissue type expressed VEGF and also that the purified VEGF product itself showed more than one distinct band at 46 kDa as expected. Further work with a monoclonal antibody to VEGF was unable to produce any bands even in the channel with purified VEGF protein product despite extensive efforts to optimise the methodology.

Discussion

The treatment of secondary hyperparathyroidism has been the subject of surgical debate since the description by Wells in 1975 of a technique involving total parathyroidectomy with re-implantation into forearm muscle. Studies have been undertaken to ascertain whether this method is preferable to sub-total parathyroidectomy (Wells et al 1975, Takagi et al 1984) where part of one parathyroid is left in situ in the patients' neck. Central to any discussion over the merits of autotransplantation is the assessment of graft function or 'take' (Saxe 1984, Brown et al 1979, Wells et al 1977) either from tissue removed and re-implanted at the time of surgery or cryopreserved parathyroid tissue (Brennan et al 1979). Thus far, no consistent system exists which clearly defines outcome in this field but common outcome measures include restoration of normocalcaemia, resolution of symptoms and no further requirement for surgery for recurrent disease (Baumann et al 1993).

Successful survival of parathyroid tissue following implantation into forearm muscle must be dependent on the development of a new blood supply from the existing microvasculature of the muscle bed to which it is grafted i.e. angiogenesis must be stimulated by the parathyroid gland itself in vivo. When trying to mimic parathyroid transplantation in vivo, a robust angiogenic response has been demonstrated by tissue derived from human samples with a histological diagnosis of hyperplasia and adenoma (Saxe 1984). This was later replicated with normal canine parathyroid tissue using an in vitro model of angiogenesis (Carter et al 1996) and then again with normal human parathyroid tissue in vitro (Carter et al 2000). Having established the presence of new blood vessel formation stimulated by parathyroid tissue in vitro, research now concentrates on the role of known mediators of angiogenesis.

It has been shown, in the same model of angiogenesis, that following explant there is a twelve fold increase in the expression of vascular endothelial growth factor

(VEGF) mRNA as shown by quantitative reverse transcription polymerase chain reaction analysis in human parathyroid cells and furthermore that the angiogenesis stimulated by this tissue is completely obliterated by the coculture with FLT-1 soluble fusion protein (Carter et al 2000). This work strongly implicates VEGF as being an important mediator of parathyroid-induced angiogenesis in this model and it has further been shown that angiopoietin-2 modulates this response (Carter et al 2001). The relevance of understanding how a parathyroid gland can survive explant and autotransplantation using an in vitro model pertains to its use in developing strategies to improve graft function following surgery. The suggestion of this work is that transplantation could take place in a favourable milieu by the systemic or local application of a 'pro-angiogenic' substance which will lead to improved outcome.

The data presented in this thesis do not directly address the subject of how human parathyroid tissue stimulates angiogenesis when transplanted but rather examines the difference in the microvasculature (and thus angiogenesis) between parathyroid tissue of differing histological type – namely normal, hyperplasia and adenoma. Evidence is presented which shows angiogenesis occurs early in the development of parathyroid disease but which is not associated with a significant increase in the number of proliferating endothelial cells, a finding which is likely to be due to technical considerations. Furthermore, using a triad of methodologies (immunohistochemistry, in situ hybridisation and Western blot analysis) to look for the expression of VEGF protein or mRNA in human parathyroid tissue, no significant increase between the differing types of tissue were observed. Indeed a counter intuitive result was obtained showing mean total VEGF immunohistochemistry scores to be higher in normal tissue than in adenoma. This suggests that in vivo, VEGF does not play a significant role in the regulation of angiogenesis in the setting of parathyroid disease.

A recent similar study has also demonstrated higher microvessel density (MVD) in parathyroid adenoma and hyperplasia versus normal gland although did not demonstrate the same step-wise progression presented here (Garcia dela Torre et al 2004). This increase in MVD correlated with an increased expression of fibroblast

growth factor 2 (FGF-2) but not with VEGF. Hence our data are supported by another study but do not concur with previous published data and it should be borne in mind that those who have demonstrated parathyroid induced angiogenesis to be VEGF dependent were using parathyroid cell culture and an in vitro assay and looking at the early response to transplantation. Furthermore it is not known whether other endocrine tissue may respond in the same way to the experimental conditions that the parathyroid cells were exposed to.

It could be postulated therefore that increased expression of VEGF may have a role in promoting angiogenesis in vivo but that this response is not sustained and must be continued by other mediators of angiogenesis. This is an area which will merit further investigation and may be best studied in parathyroid tissue that has been implanted and functioning.

Although it has been demonstrated that parathyroid proliferative lesions demonstrate evidence of angiogenesis it is likely that this progression to an angiogenic phenotype occurs through differing pathways. This is evidenced in part by the discovery that genes up-regulated in parathyroid adenoma are those responsible for angiogenesis and production of blood vessels whereas genes down-regulated in adenoma but expressed in parathyroid hyperplasia are related to a decrease in apoptosis (Schachter et al 2002). This study concluded that the two are different physiologic conditions and that further analysis of kinase genes involved in angiogenesis and apoptosis will enable targeting of the key genes responsible for the development of hyperplasia and adenoma.

Chapter 4: Validation studies - assessment of observer variation and reproducibility of microvessel counts in renal biopsy specimens

This section presents the results of the experiments described in sections 2i and 2ii in the Materials and Methods chapter which were:

- assessment of observer variation by comparison of microvessel counts in normal kidney between two observers and at two time points by one observer (section 2i).

- comparison of microvessel counts between core biopsy and cross section pairs from normal kidney (section 2ii),

- comparison of microvessel counts between core biopsy and cross section pairs from kidney tumour

- comparison of microvessel counts between cross sections and biopsies from normal kidney and tumour from the same patient

The first two experiments are of direct relevance to the later studies described below. Reproducibility of microvessel counts by the principal investigator must be demonstrated in order for that technique to be confidently employed in later experiments comparing MVCs between normal and CAN kidney (chapter 5).

Validation of the use of MVCs in renal biopsy specimens as being representative of MVCs in kidney cross section specimens is necessary in order to use renal allograft

specimens in the study of the progression of vascular changes during CAN (chapter 6+7).

The study of MVCs in renal tumour specimens is of less direct relevance to further studies presented here. These data are included for two reasons:

- they provide confirmation that normal kidney microvasculature studied from kidneys with coexisting tumours and used as control is unaffected by the adjacent tumour

- they demonstrate consistency in methodology when studying both normal and diseased kidney.

Results

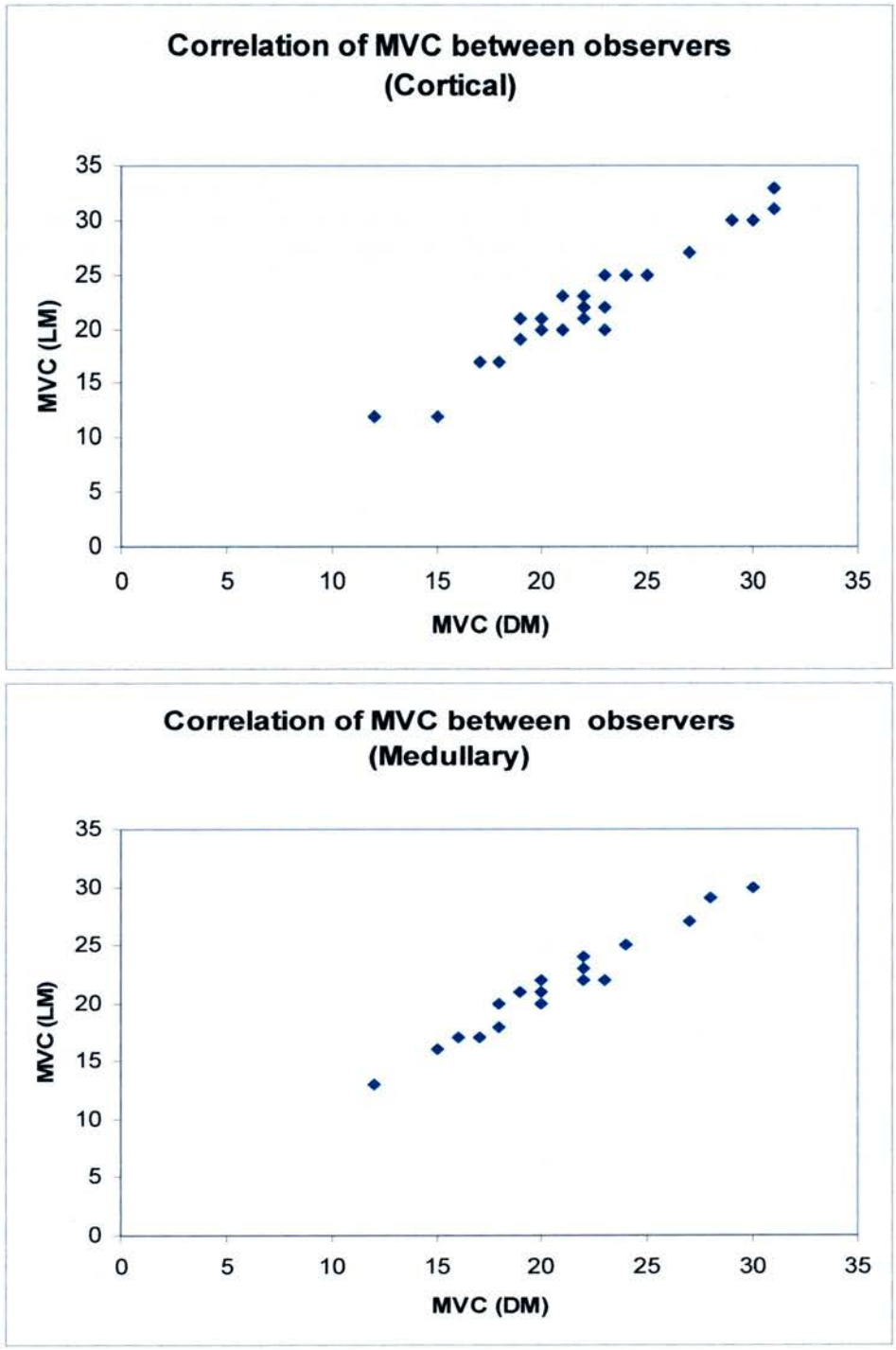
Inter-observer and sampling variation

Microvessel counts were performed separately by two observers (mvcDM and mvcLM) to assess inter-observer variation (Figure 4.1). Counts were performed with a conference microscope with each observer looking at the same fields from the same section at the same time and recording scores. These were then compared once all counts had been performed.A significant correlation was found between the counts of the two observers (Pearson correlation: cortex $r=0.8844$ with $p<0.0001$, medulla $r=0.98$ with $p<0.0001$).

Microvessel counts were performed by observer DM at two separate time points one month apart (mvcDM1 and mvcDM2) to assess for sampling variation and intra-observer variation (Figure 4.2). The counts were obtained from the same sections but in differing fields each time. Thus variation in counts may occur either due to a change in technique by observer DM or by virtue of examining different fields within each section. However a significant correlation was found between the two counts from observer DM which demonstrates that the method is not susceptible to sampling variation or intra-observer variation (Pearson correlation: cortex $r=0.68$ with $p<0.0001$, medulla $r=0.61$ with $p=0.0002$).

Pictures of CD31 staining of normal kidney cortex and medulla will be presented in the next chapter.

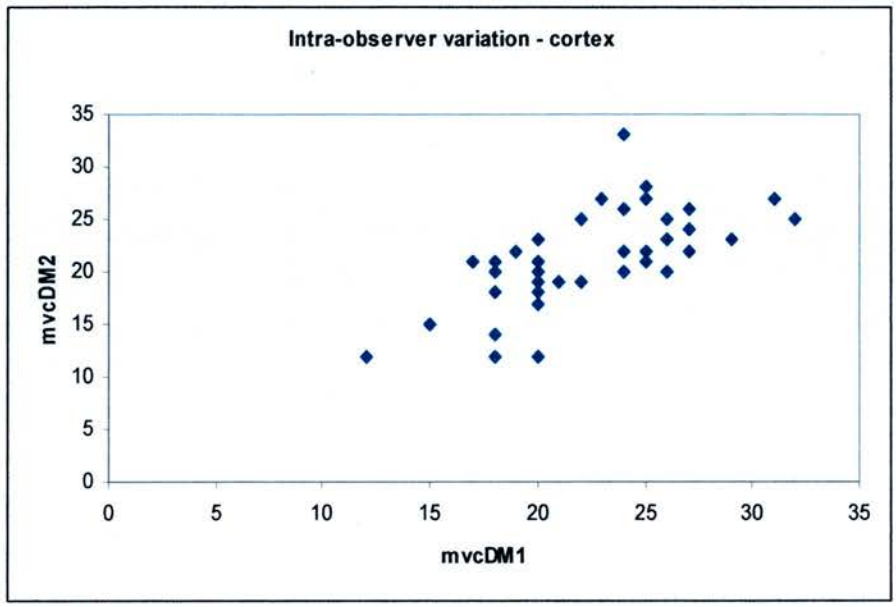
Figure 4.1 – Inter-observer variation: LM vs. DM

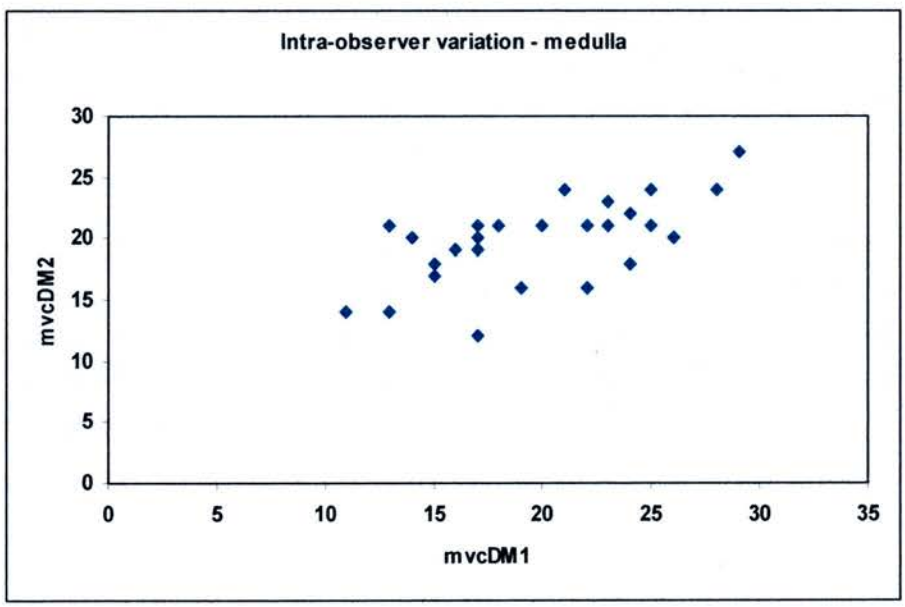


Cortical staining, r=0.8844 with p<0.0001. (Pearson correlation)

Medullary staining, r=0.98 with p<0.0001 (Pearson correlation)

Figure 4.2 – Sampling and intra-observer variation: DM1 vs. DM2





Cortical staining, r=0.68 with p<0.0001. (Pearson correlation)

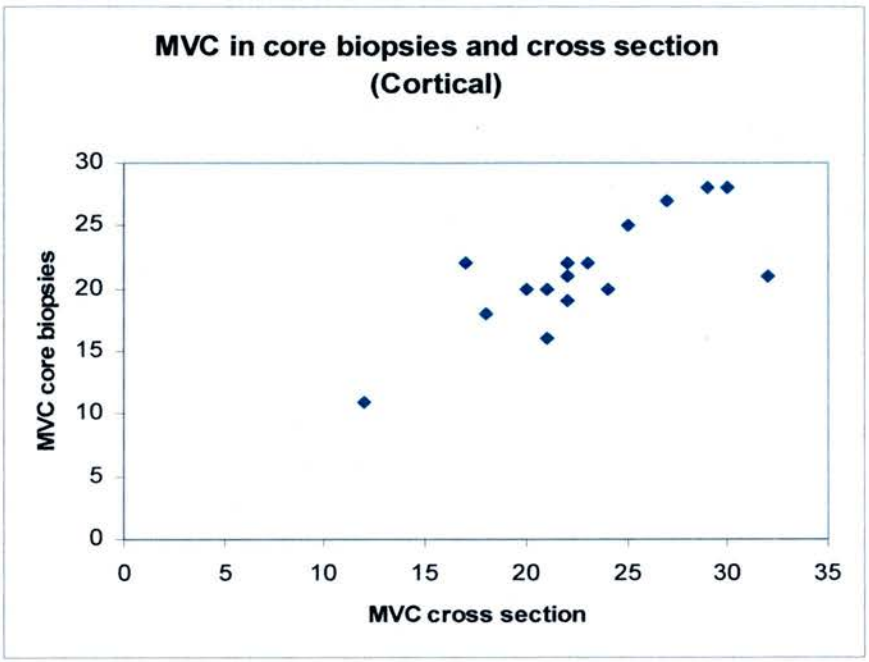
Medullary staining, r=0.61 with p=0.0002 (Pearson correlation)

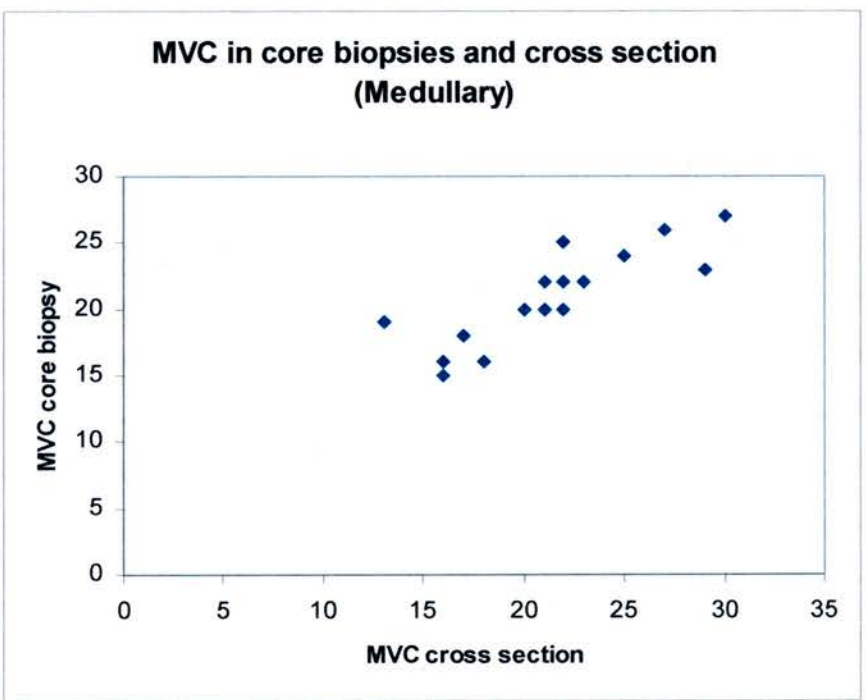
Comparison of microvessel counts between core biopsy and cross section pairs from normal kidney

Microvessel counts were performed on 25 of the 26 specimens of normal kidney and kidney biopsy. One specimen did not stain for CD31 and was therefore unsuitable for analysis. Due to variations in specimen collection not all 25 samples provided both cortical and medullary results. 20 pairs of cortical counts and 17 pairs of medullary counts were obtained and all biopsy specimens included in the analysis of cortical counts had greater than 7 glomeruli, defined as adequate using Banff classification (Racusen 1997).

Cortical and medullary MVCs for the paired cross-section (xs) and biopsy (bx) demonstrate a highly significant positive correlation (cortical counts, $r=0.7658$, $p<0.0001$; medullary counts, $r=0.8429$, $p<0.0001$)(Figure 4.3).

Figure 4.3 – Correlation between Normal Biopsy MVC and Cross Section MVC (same kidney)





Cortical staining, r=0.7658 with p<0.0001. (Pearson correlation)

Medullary staining, r=0.8429 with p<0.0001. (Pearson correlation)

Microvessel counts were performed on all of the 26 specimens of kidney tumour and biopsy. Several specimens did not stain for CD31 and were therefore unsuitable for analysis. Due to variations in specimen collection not all 26 samples provided results. 20 cross section counts and 20 biopsy counts were obtained and all biopsy specimens included in the analysis of cortical counts had greater than 7 glomeruli, defined as adequate using Banff classification (Racusen 1997).

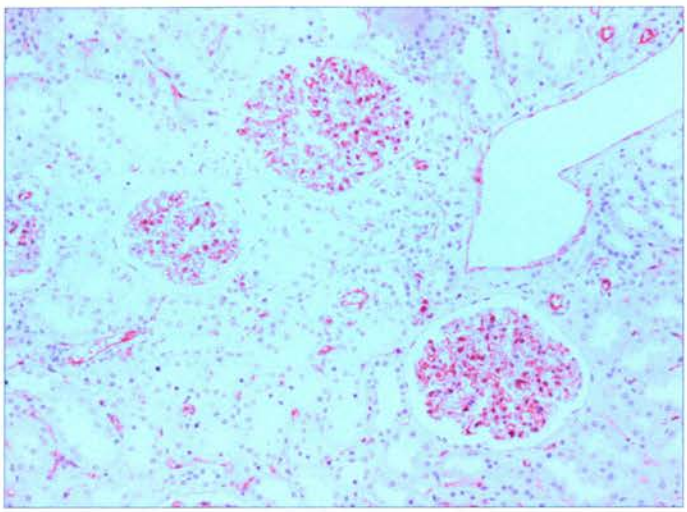
Heterogeneity of vasculature is exhibited in certain human tumours and microvascular 'hot spots' are seen e.g. breast cancer (Weidner et al 1991, Van Hoef et al 1993). If such heterogeneity existed in renal cell carcinoma then it would be anticipated that core biopsy specimens may 'miss' these hot spots and give lower microvessel counts than the corresponding tumour cross section.

The architecture of tumour sections and vascularity was distorted compared to normal kidney as shown above in Figure 4.4.

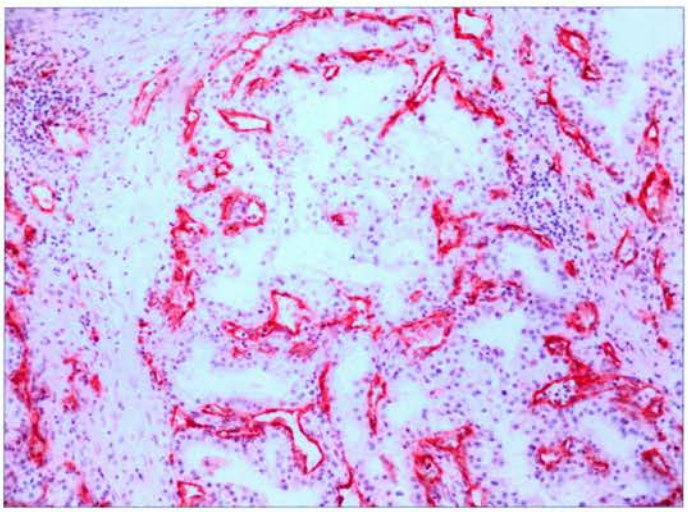
Figure 4.5 shows MVCs for the paired tumour cross-section (xs) and biopsy (bx) demonstrating a highly significant positive correlation (counts, $r=0.7450$, $p=0.0002$).

This interesting observation implies that there exists a homogeneity of microvasculature in the renal tumours studied here.

Figure 4.4: Comparison of CD31 staining in normal kidney vs. renal tumour

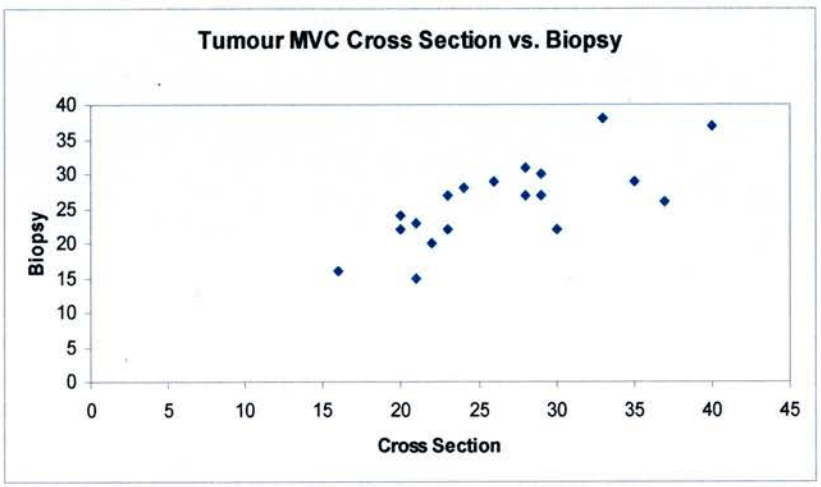


Normal renal cortex (above) with clearly visible glomeruli and no distortion of architecture.



Renal tumour with no glomeruli visible and marked CD31 immunostaining.

Figure 4.5 – Correlation between Tumour Biopsy MVC and Tumour Cross Section MVC (same kidney)



Tumour staining, r=0.7450 with p = 0.0002. (Pearson correlation)

Comparison of microvessel counts between cross sections and biopsies of normal kidney and tumour from the same patient

Microvessel counts were performed on 25 of the 26 specimens of normal kidney and all 26 specimens of tumour. Several specimens did not stain for CD31 and were therefore unsuitable for analysis. Due to variations in specimen collection not all 25 samples provided results. 20 cross section counts and 17 biopsy counts were obtained.

Table 4.1 – MVC in normal kidney and renal tumour

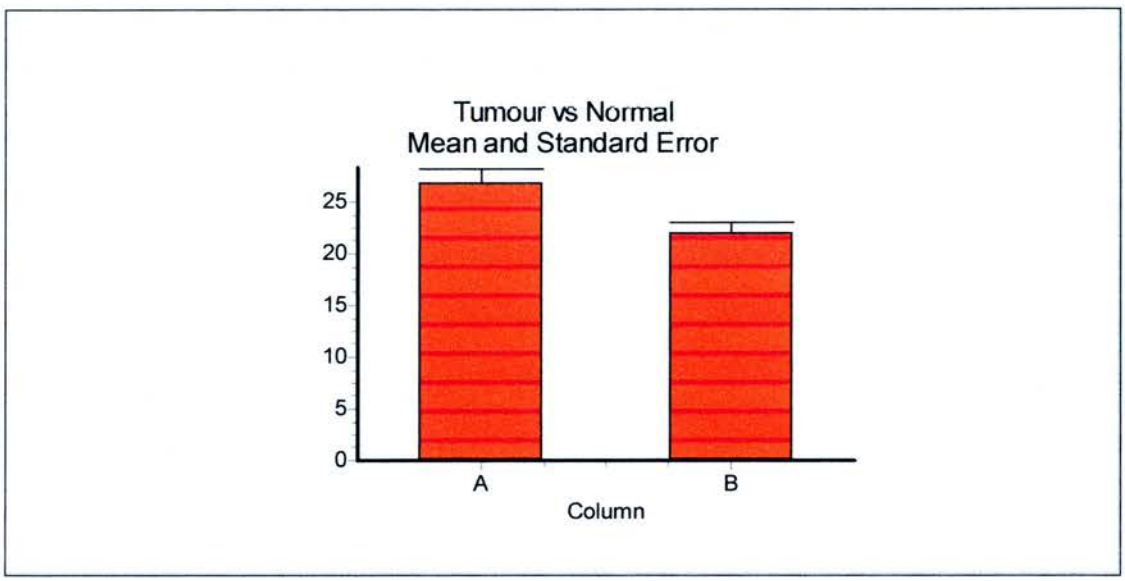
	Cross section	**Biopsy**
Normal cortex	**22.0 (12 – 31)** 4.95	**20.9 (11 – 28)** 3.96
Normal medulla	**20.1 (13 -30)** 4.02	**20.8 (15 – 27)** 3.57
Tumour	**26.9 (20 -40)** 5.80	**26.1 (15 – 38)** 5.56

Mean (Range) Standard deviation

Tumour vs. normal cortex: $p = 0.0040$ (Paired t-test)

Tumour vs. normal medulla: $p<0.0001$ (Paired t-test)

Figure 4.6 – Correlation between Tumour MVC and Normal Kidney MVC (same kidney)



A – Tumour MVC

B – Normal MVC

There is significant increase in tumour MVC (26%) compared with the surrounding normal kidney. Comparing mean tumour MVC with mean normal (cortex and medulla combined) MVC $p = 0.0347$.

Discussion

The present study has attempted to assess the variation in assessment of angiogenesis in kidney sections which might be caused by lack of reproducibility of methodology or heterogeneity of tissue.

Tissue heterogeneity of microvasculature was investigated in the present study by comparing microvessel counts in renal core biopsies and cross-sections taken simultaneously. Counts were performed for both the cortex and medulla of each section and a statistically significant correlation between the two counts was achieved. This supports the use of microvessel counts in renal biopsy material in the assessment of angiogenesis as being representative of the kidney as a whole and further suggests that there exists a homogeneity of vasculature in the kidneys studied here and that the phenomenon of vascular 'hot spots' is not present in this tissue as has been shown in human tumours (Weidner et al 1991, Van Hoef et al 1993). The wider implication of these results is that they will allow for the use of microvessel counts from renal biopsy specimens where this offers the only available source of tissue to study and to draw inference about the kidney from which the biopsy was taken.

There was a highly significant correlation between counts performed by two different observers and between two time points for one observer. Previous studies that have addressed the issue of observer variation when performing microvessel counts have advocated the use of a period of training with an experienced observer (Vermeulen et al 1997), the use of a Chalkley eyepiece graticule (Fox et al 1995, Marson et al 1999) and the use of a conference microscope (Hansen et al 1998). All of these factors have been included in this present study to minimise observer variability and our results are comparable with those published (Vermeulen et al 1997, Fox et al 1995, Marson et al 1999).

This present study has employed an artificial technique of specimen collection in order to acquire sufficient samples of paired biopsies and cross sections taken simultaneously from the same kidney. The authors accept that this method only mimics that seen in clinical practice where the percutaneous collection of renal biopsy material is performed.

Furthermore, some of the kidney which has been studied has been taken from organs in which a renal tumour co-exists which may have the effect of distorting the 'normal' kidney vasculature however sections were confirmed by a pathologist to be normal kidney. Microvessel counts were also performed on specimens of tumour taken simultaneously with the normal kidney and shown to be significantly higher (26% increase in MVC, $p=0.0347$) suggesting that although the tumour vasculature has expanded this effect is not experienced by the surrounding non-tumour kidney.

In summary, accepting the limitations of the study design as dictated by the constraints of the source of tissue used, this study addressed the validity of using microvessel counts from renal biopsies to represent the kidney as a whole and also the practical issues regarding this methodology. Observer variation of counts was similar to the results of other studies in other tissues as a result of the use of experienced observers, a Chalkley eyepiece graticule and a conference microscope. Homogeneity of kidney microvasculature has been demonstrated using this reproducible method which will allow future investigators to use biopsy as a more readily available source of renal tissue in the investigation angiogenesis in renal disease. These data have also allowed quantification of the expansion of microvasculature that occurs in renal tumours.

Chapter 5: Changes in vascularity in allograft nephrectomy specimens with chronic allograft nephropathy

In the previous chapter it was demonstrated that immunostaining of renal tissue with antibody to CD31 followed by performing microvessel counts (MVCs) was a reproducible methodology in experienced hands and with the appropriate technique.

Having validated the use of this methodology it was possible to perform MVCs on nephrectomy specimens performed because of chronic allograft nephropathy (CAN) and compare these with normal kidney.

As will be presented below, there was a significantly lower MVC in the CAN group suggesting loss of microvasculature during the development of CAN. Further investigation was undertaken to seek an association of this finding with endothelial cell proliferation and macrophage infiltration within the allografts.

Results

This section presents results obtained using three immunohistochemical staining methods to compare normal kidney with nephrectomies performed because of CAN. Examples of each staining method are presented on the next pages.

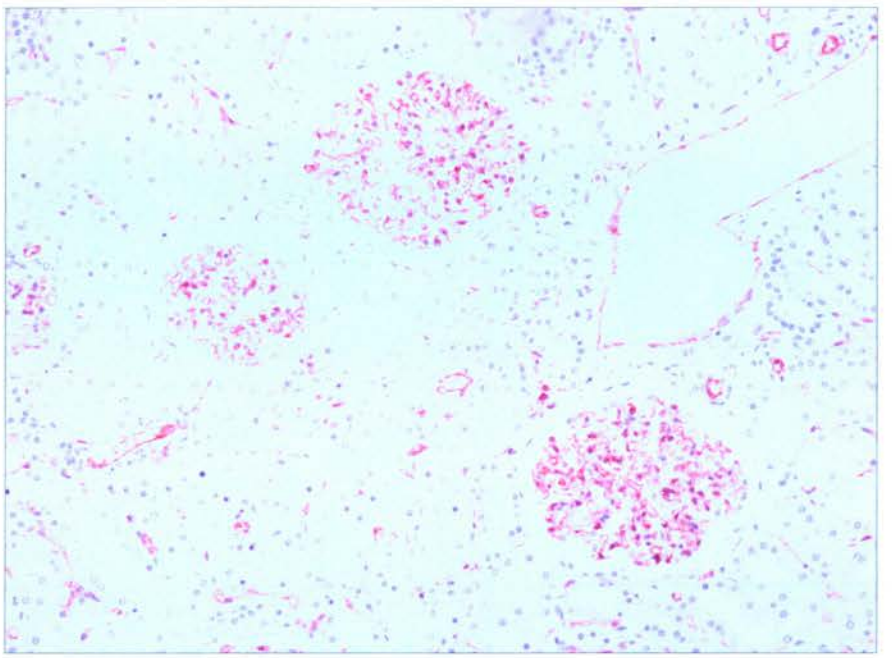
• Figure 5.1 shows representative samples of the immunohistochemical analysis of normal renal tissue stained with the CD31 antibody.

• Figure 5.2 shows cortical and medullary sections from normal and CAN kidney stained with antibody to CD31.

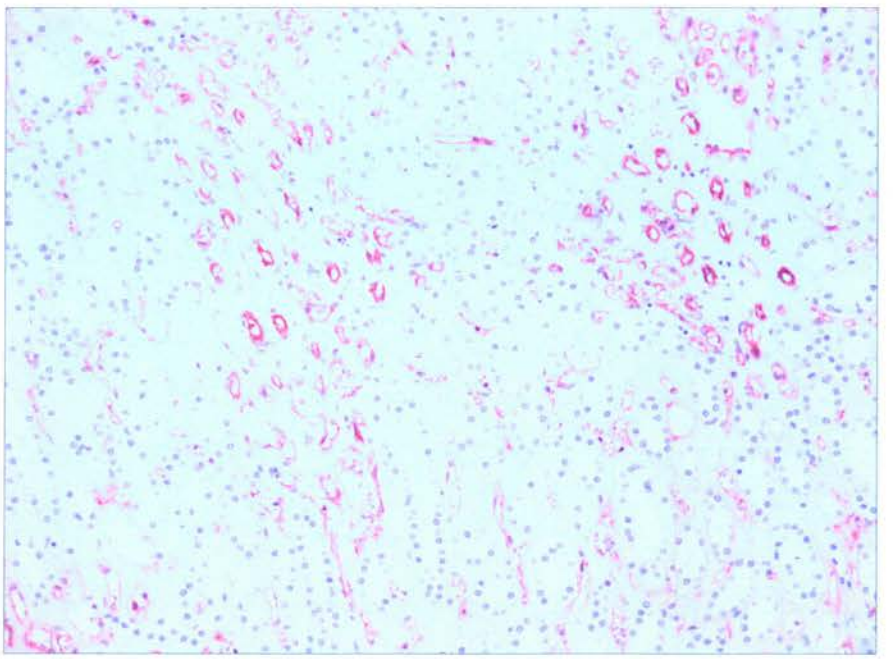
• Figure 5.3 shows an example of dual CD31/MIB-1 staining with a proliferating endothelial cell marked. This image is from parathyroid tissue as this most clearly demonstrates the appearance of a proliferating endothelial cell also seen in the kidney specimens.

• Figure 5.4 shows an example of CD68 staining with a glomerular macrophage marked.

Figure 5.1; Normal kidney CD31 staining cortex and medulla

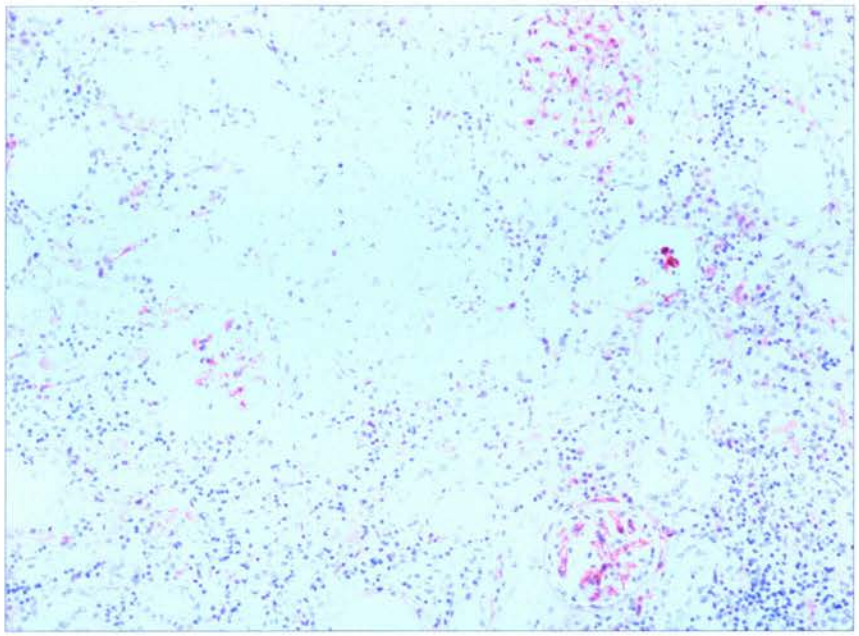


Normal cortex (above) showing three normal glomeruli and a prominent vessel in the top right corner.

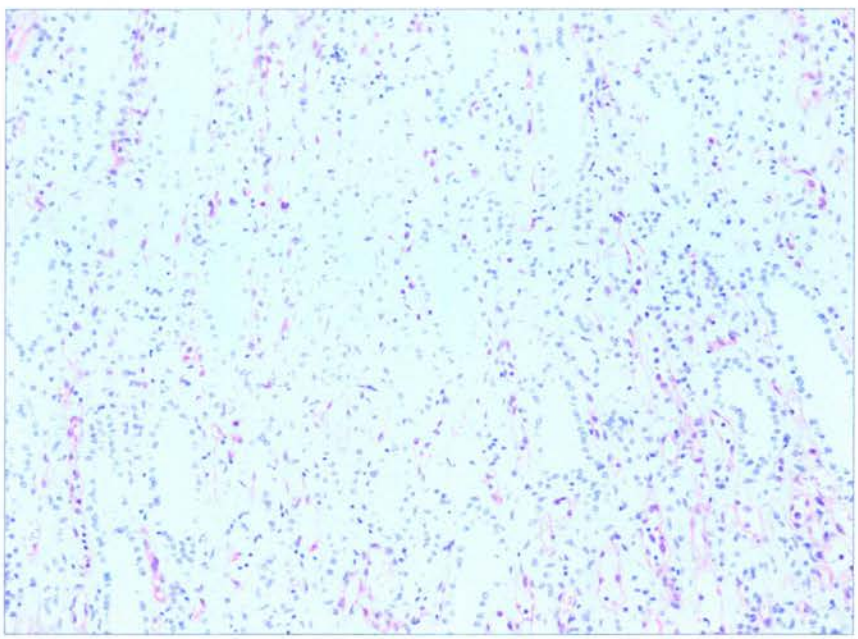


Normal medulla (above) showing healthy tubules and normal regular vasa recta staining pink.

Figure 5.2 – CD31 staining of CAN nephrectomy

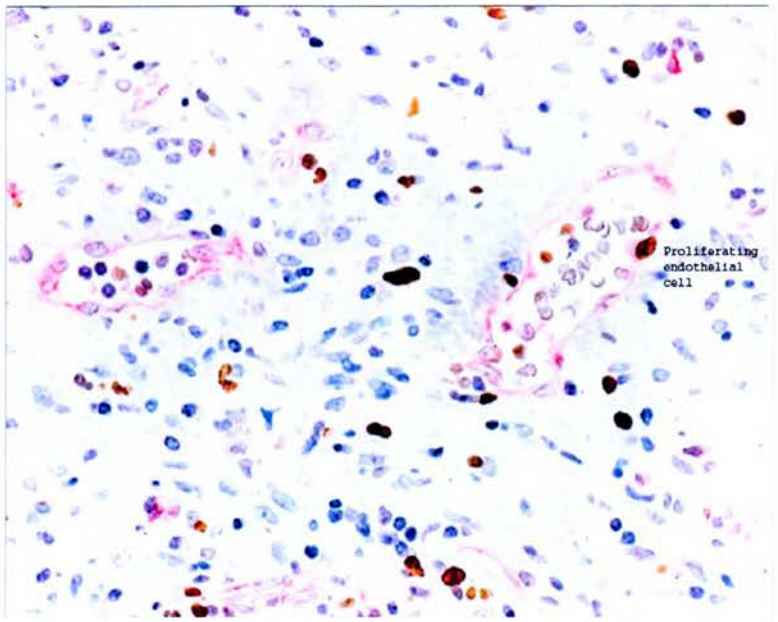


Cortex of nephrectomy with CAN. There is loss of normal architecture within the glomeruli and a reduction in CD31 staining compared with the normal cortex.



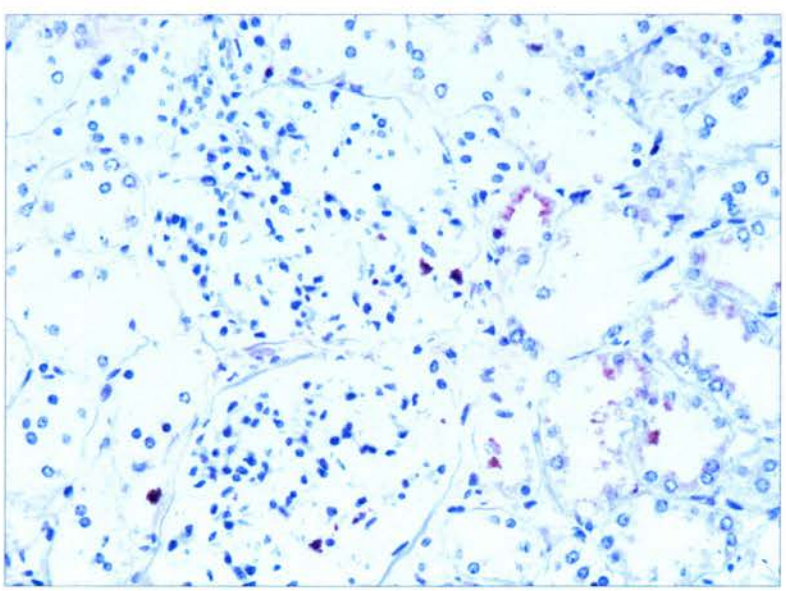
Medulla of nephrectomy with CAN. There is tubular loss, some fibrosis and marked loss of vasa recta compared with normal.

Figure 5.3 – Example of proliferating endothelial cell



The dual stained (brown and pink) proliferating endothelial cell is marked at the top right of the image. This image is taken from parathyroid adenoma and demonstrates two main vessels to the left and right of the image (pink staining) and evidence of cellular proliferation (brown staining cells seen throughout the image).

Figure 5.4 – Example of glomerular macrophage



Two glomerular macrophages (brown) are present in the centre of the image.

Comparison between MVC of normal kidney and CAN nephrectomy

There was a loss of regularity of CD31 staining in CAN nephrectomies compared with normal (Figure 5.2). Due to variations in intensity of staining, tissue availability and small specimen size, not all samples provided both cortical and medullary results. Cortical microvessel counts were obtained from 29 nephrectomies performed because of CAN and 61 specimens of normal kidney. Medullary microvessel counts were obtained from 28 CAN nephrectomies and 45 specimens of normal kidney.

Table 5.1 and Figure 5.5 show a significant reduction in MVC in the CAN nephrectomy group compared with normal kidney ($p<0.0001$ and $p<0.0001$ for cortex and medulla respectively).

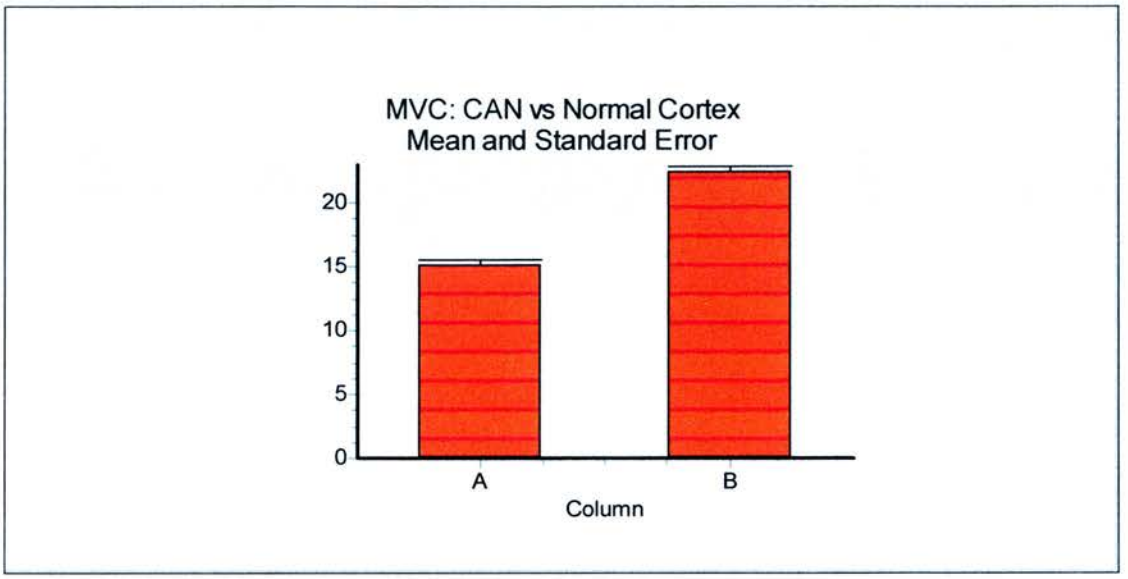
Table 5.1 – MVC in Normal Kidney vs. CAN Nephrectomy

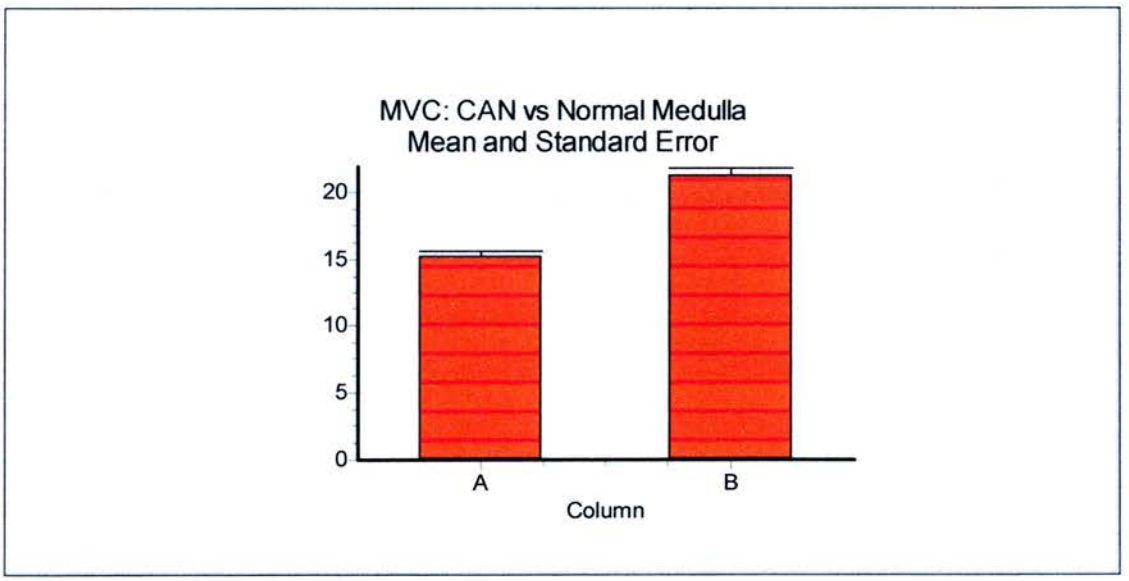
	Normal cortex	CAN cortex	Normal medulla	CAN medulla
Mean	22.36	15.08	21.31	15.15
Standard deviation (SD)	4.20	2.61	3.94	2.87
Standard error of mean (SEM)	0.45	0.44	0.48	0.50
Range	12 - 32	11 - 22	13 - 30	11 - 23

Cortex:Mean MVC Normal vs CAN: $p<0.0001$ (Paired t-test)

Medulla: Mean MVC Normal vs CAN: $p<0.0001$ (Paired t-test)

Figure 5.5 – Mean MVC CAN Nephrectomy vs. Normal Kidney





A – CAN

B - Normal

Comparison between no. of proliferating endothelial cells in normal kidney and CAN nephrectomy

Following dual staining with CD31 and MIB-1, scores were obtained from 18 CAN nephrectomies and 11 normal kidneys and results presented below (Table 5.2 and Figure 5.6).

A significantly higher number of proliferating endothelial cells were present in the CAN nephrectomy group versus normal ($p<0.0001$).

The proliferation index in the CAN nephrectomy group was significantly higher than in normal kidney ($p<0.0001$).

Thus, despite a significantly lower MVC, the CAN group have a significantly higher number of proliferating endothelial cells compared to normal kidney.

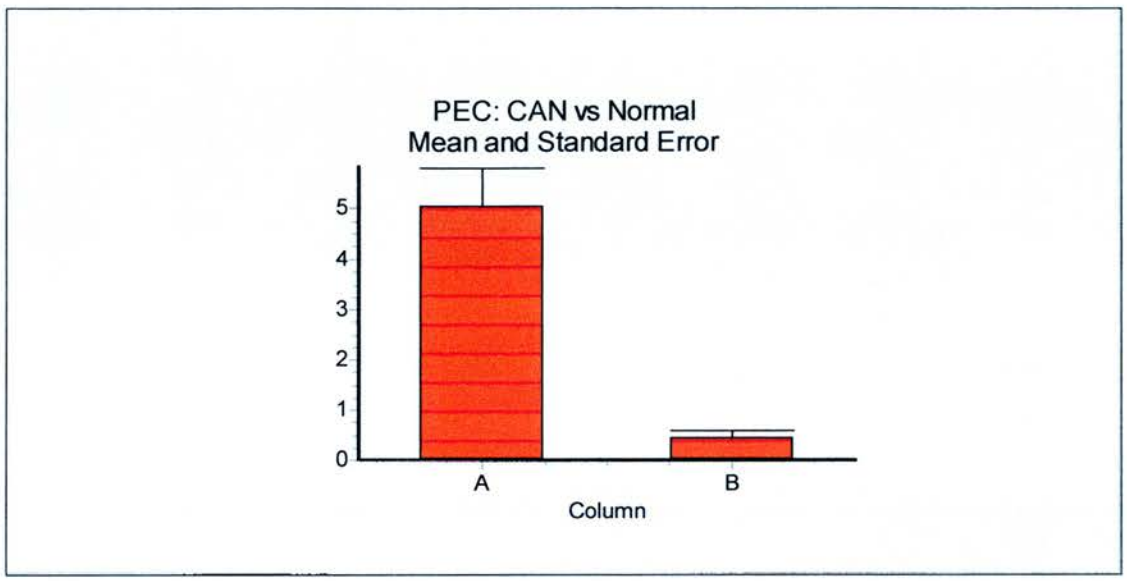
Table 5.2 – Number of Proliferating Endothelial Cells (PECs) and Proliferation Index (PI) in Normal kidney vs. CAN Nephrectomy

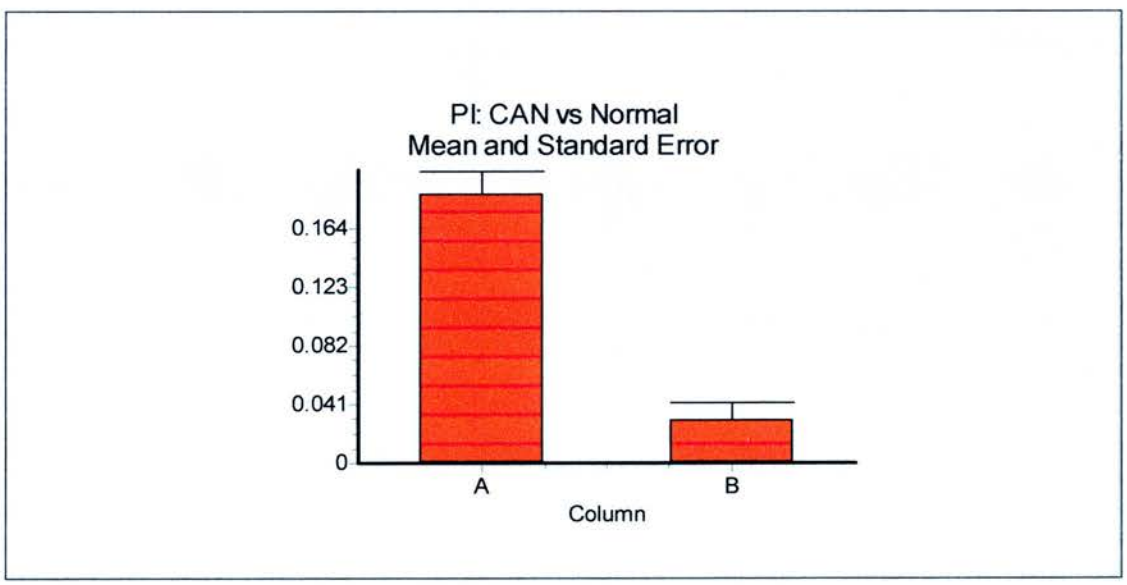
	Normal PEC	CAN PEC	Normal PI	CAN PI
Mean	0.44	5.05	0.03	0.19
Standard deviation (SD)	0.63	3.17	0.044	0.066
Standard error of mean (SEM)	0.16	0.74	0.011	0.016
Range	0 - 2	1 – 15	0 – 0.12	0.07 – 0.30

Normal vs. CAN: $p<0.0001$ for mean no. of proliferating ECs (Student t-test)

Normal vs. CAN : $p<0.0001$ for mean PI (Student t-test)

Figure 5.6 - Number of Proliferating Endothelial Cells (ECs) and Proliferation Index (PI) in Normal kidney vs. CAN Nephrectomy





A – CAN

B - Normal

Comparison between number of glomerular macrophages in normal kidney and CAN nephrectomy

Following staining with the antibody to CD68, the number of glomerular macrophages were counted in several high power (x250) fields. Where possible 20 glomeruli were counted for each section studied.

Sections from 18 normal kidneys and 29 nephrectomies performed because of CAN were studied.

The glomerular macrophage index (GMI) was calculated as;

GMI = Total no. of glomerular macrophages / No. of glomeruli studied per section

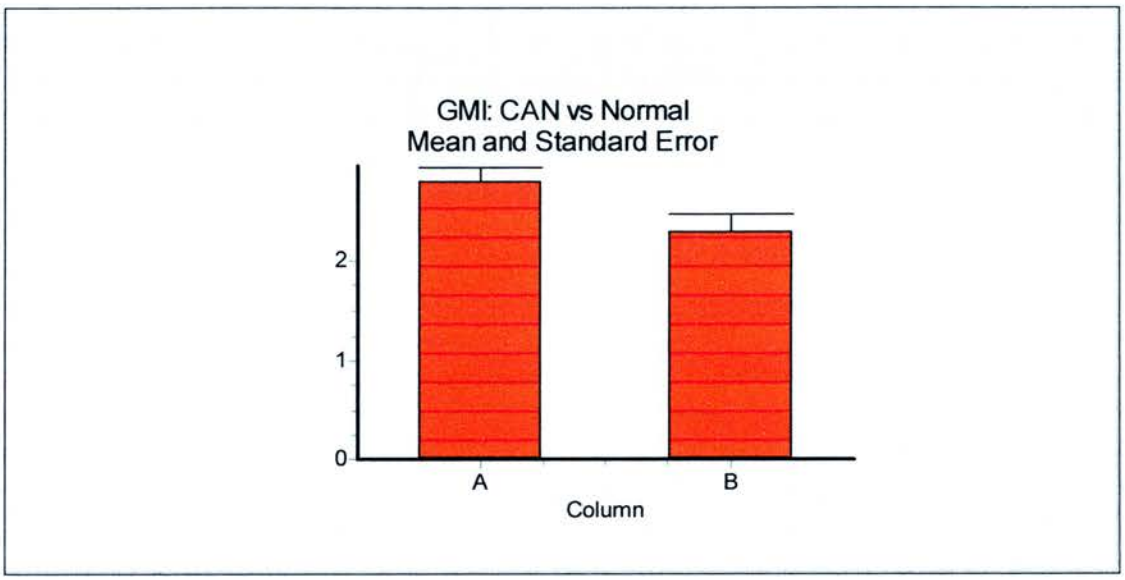
Table 5.3 and Figure 5.7 show that despite a trend for CAN nephrectomies to have a higher GMI this was not statistically significant.

Table 5.3 – Comparison of GMI in Normal Kidney and CAN Nephrectomy

	Normal GMI	**CAN GMI**
Mean	2.29	2.80
Standard deviation (SD)	0.80	0.74
Standard error of mean (SEM)	0.18	0.14
Range	1.25 – 3.10	1.55 – 3.95

Mean GMI: Normal vs. CAN: $p = 0.0368$ (Unpaired t-test). There is a significantly higher GMI in samples of CAN nephrectomy specimens than normal kidney.

Figure 5.7 – Comparison of mean GMI between normal kidney and CAN nephrectomy



A – CAN

B - Normal

Discussion

These results show that end stage CAN is associated with significant loss of microvasculature as shown by the reduced microvessel count in this group. This loss of microvessels has occurred despite evidence ongoing attempts at endothelial repair as demonstrated by the presence of proliferating endothelial cells in the CAN group.

An explanation for these findings is that despite the presence of end stage CAN there remains a stimulus within the graft to repair endothelial cells and restore normal vasculature i.e. angiogenesis. Further studies of levels of expression of known mediators of angiogenesis (e.g. VEGF) in this tissue may confirm that this is the case.

There was a statistical significant increase in numbers of glomerular macrophages in CAN compared with normal. The relevance of this finding is uncertain as cessation of immunosuppressive therapy in the CAN group is likely to be associated with an increase in macrophage number. Alternatively the higher number of macrophages may represent a cause for microvessel loss via macrophage mediated endothelial cell killing.

These findings support a hypothesis that early following transplantation there is stimulation of angiogenesis but that with progression of CAN, microvessel loss predominates. Evidence that persistent attempts at tissue repair (manifested by the presence of proliferating endothelial cells) are ongoing even with end stage disease may be of great clinical relevance and could provide an area for novel therapeutic intervention.

The following section of this thesis examines these changes in vasculature and endothelial cell proliferation by studying biopsies from the grafts studied in this section which developed CAN.

Studies that were beyond the scope of this thesis which would be of future interest include:

• assessment of the expression of specific mediators of angiogenesis during development of CAN and the correlation with MVC

• a dynamic assessment of endothelial cell proliferation and death and a correlation with both MVC and the expression of specific mediators of angiogenesis.

Chapter 6: Changes in vascularity associated with chronic allograft nephropathy using allograft biopsies

Results from the previous chapter demonstrated a significant reduction in microvessel count (MVC) in end stage CAN to be associated with a significantly higher number of proliferating endothelial cells when compared with normal kidney. A possible hypothesis to explain this finding is that angiogenesis is stimulated following transplantation but that with progression of CAN microvessels are lost despite attempts at repair.

In order to examine this finding further, biopsy specimens from some of the allografts that developed CAN were studied and similar immunohistochemical analyses performed.

The validity of measuring MVCs in kidney biopsies to assess vascularity of the kidney as a whole has been confirmed and results presented in Chapter 4.

The biopsy specimens were taken because of clinical indication at a variable time after transplantation - mean 24 months, median 17 months after transplantation - and at a variable time prior to nephrectomy (details included in Materials and Methods section). At the time of biopsy all allografts had some degree of function and all patients were receiving calcineurin inhibitor immunosuppressive therapy.

By comparing each biopsy with the paired nephrectomy specimen it was possible to assess earlier changes in vascularity that occur during the progression of CAN which would give an indication as to the dynamic changes that occur.

Results

Comparison between MVC of CAN nephrectomy and clinical biopsy

Due to variations in intensity of staining, tissue availability and small specimen size, not all of these samples provided both cortical and medullary results.

Microvessel counts were first compared between normal kidney and clinic biopsies to assess earlier changes in the microvasculature. These were unpaired samples. A paired comparison was then made between biopsy and nephrectomy specimens from the same graft. As previously these data are presented for cortex and medulla.

A significant reduction in cortical and medullary MVC was seen comparing normal kidney with the biopsy specimens from kidneys that went on to develop CAN (Table 6.1 and Figure 6.1).

As detailed in Table 6.2 and Figure 6.2, a significant reduction in MVC was shown in the CAN nephrectomy group compared with the antecedent/clinical biopsies.

These data confirm that early in the development of CAN there is some preservation of microvasculature with significantly higher numbers of microvessel present in the biopsy group. Again an association of this finding with endothelial cell proliferation and macrophage infiltration has been sought.

Table 6.1 – MVC in Normal kidney vs. Clinical Biopsy

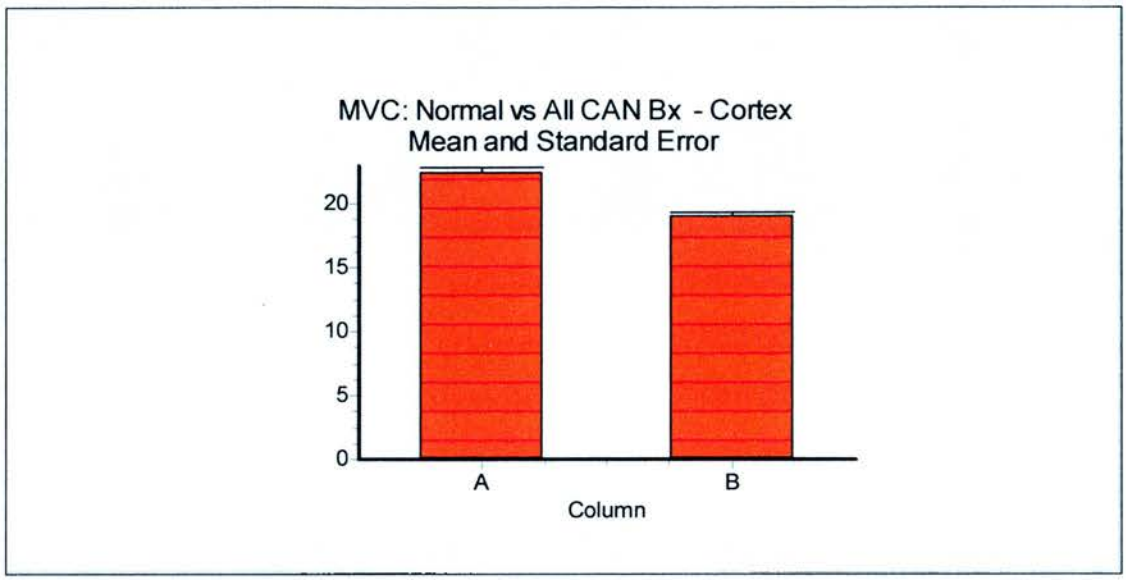
	Normal cortex	All CAN Bx cortex	Normal medulla	All CAN Bx medulla
Mean	22.36	19.00	21.31	18.34
Standard deviation (SD)	4.21	2.03	3.94	2.89
Standard error of mean (SEM)	0.45	0.35	0.48	0.54
Range	12 – 32	13 – 23	13 – 30	12 - 25

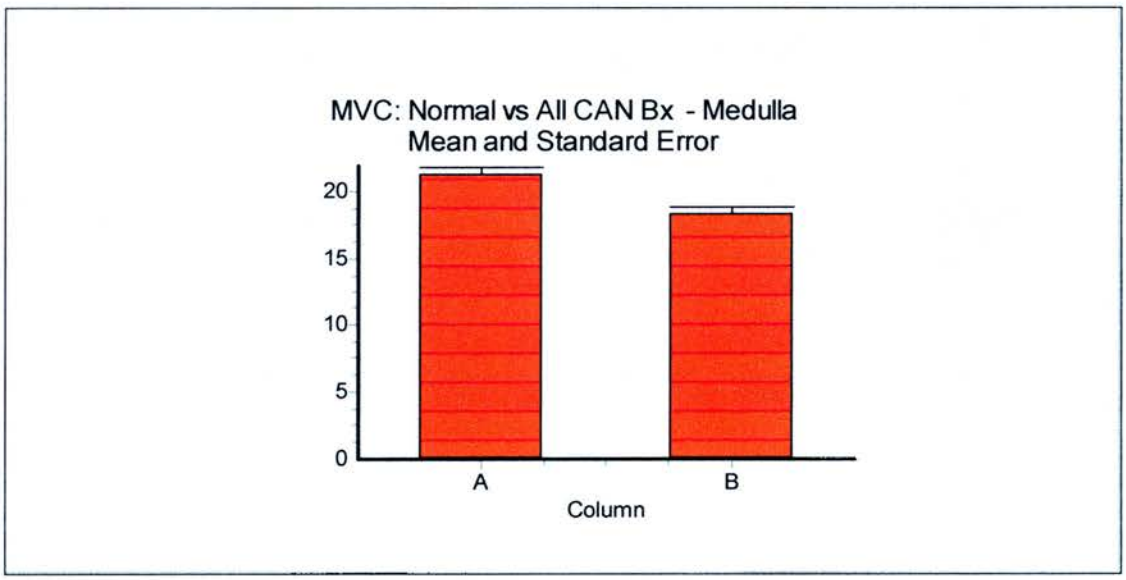
Mean cortical MVC: Normal vs. CAN Bx: $p<0.001$ (unpaired t-test)

Mean medullary MVC: Normal vs. CAN Bx: $p<0.001$ (unpaired t-test)

There is a significant reduction in MVC in the biopsy specimens taken from grafts which develop CAN.

Figure 6.1 – Mean MVC Normal Kidney vs All CAN Bx (Cortex and Medulla)





A - Normal

B – CAN Bx

Table 6.2 – MVC in Specific Clinical Biopsy and CAN Nephrectomy Pairs (same graft)

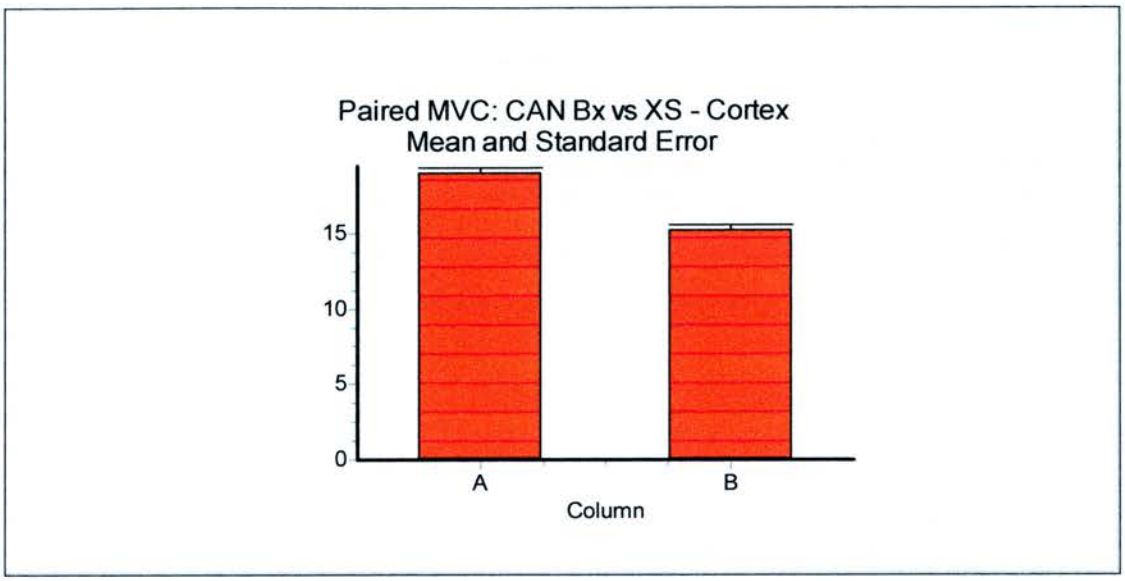
	CAN Bx cortex	CAN XS cortex	CAN Bx medulla	CAN XS medulla
Mean	19.00	15.18	18.34	15.28
Standard deviation (SD)	2.03	2.41	2.89	2.64
Standard error of mean (SEM)	0.35	0.42	0.54	0.49
Range	13 – 23	12 - 18	12 - 25	11 – 19

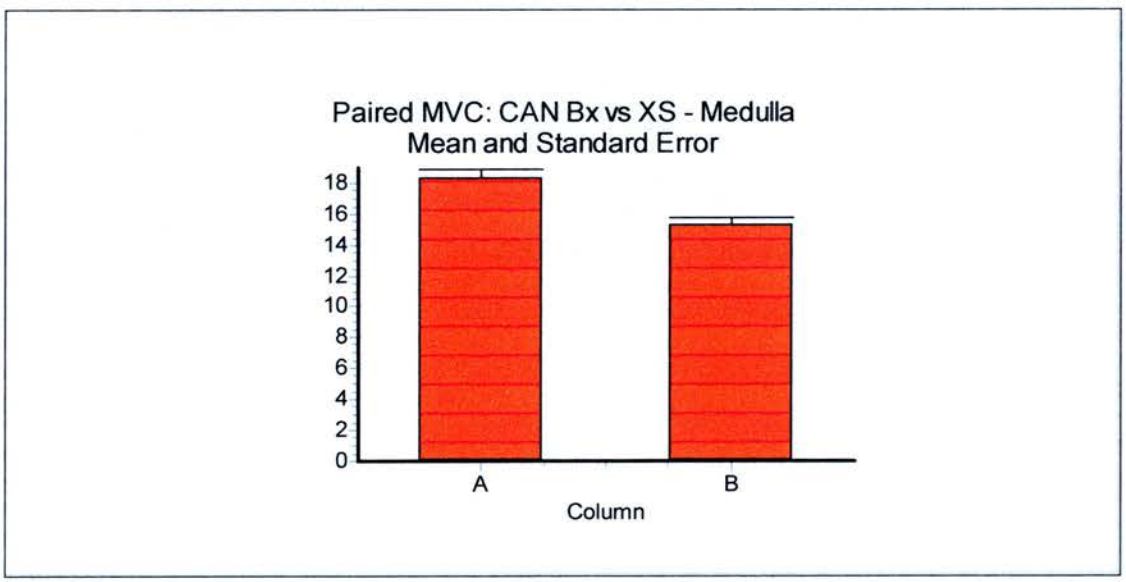
Cortical MVC: Paired CAN Bx vs CAN XS: $p<0.0001$ (Paired t-test)

Medullary MVC: Paired CAN Bx vs CAN XS: $p=0.0002$ (Paired t-test)

MVC in biopsy specimens from grafts which develop CAN are significantly higher when compared with the MVC from the same nephrectomy specimen.

Figure 6.2 – Mean MVC Paired Clinical Biopsy vs. CAN nephrectomy (cortex and medulla)





A – CAN Bx

B – CAN XS

Comparison between number of proliferating endothelial cells in CAN nephrectomy and clinical biopsy

Following dual staining with CD31 and MIB-1, scores were obtained from 18 CAN nephrectomies, 14 biopsies and 11 normal kidneys.

Table 6.3 and Figure 6.3 demonstrate that a significantly higher number of proliferating endothelial cells were present between the biopsy group and normal ($p<0.0001$). This is relevant in that it confirms that earlier in the development of CAN there are ongoing attempts at tissue repair.

Comparison between the biopsy specimens and the nephrectomies was performed to determine whether there was a significant difference in the degree of endothelial cell proliferation at different time points in CAN. Table 6.4 and Figure 6.4 confirm that there is no significant difference in the number of proliferating endothelial cells was observed between the nephrectomy and biopsy groups ($p=0.471$ - paired t-test).

These data demonstrate that proliferating endothelial cells are present in allograft biopsy specimens early in the development of CAN and at levels greater than seen in normal kidney.

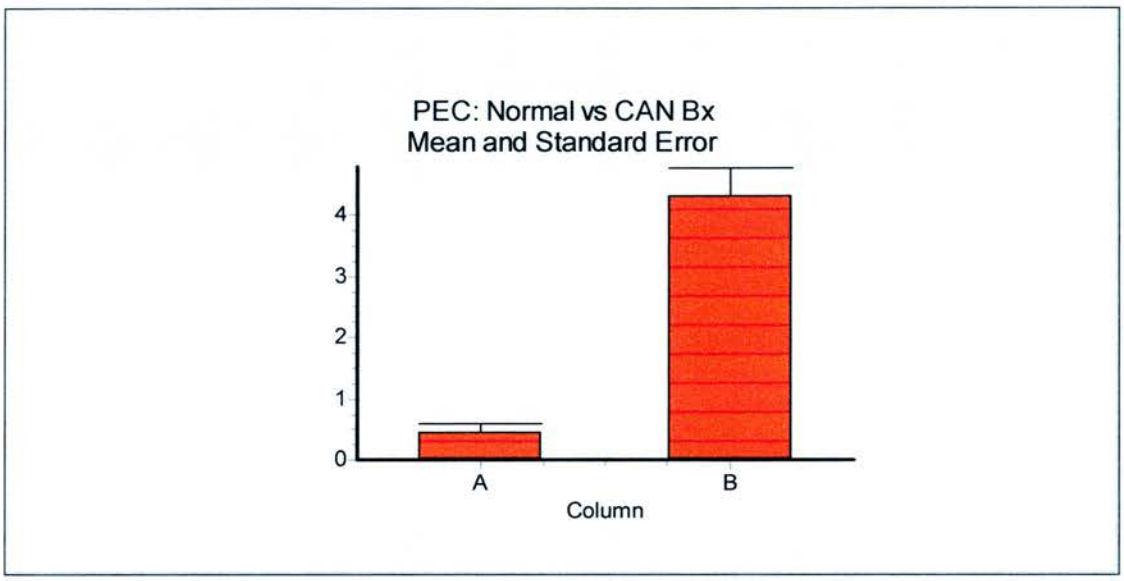
Table 6.3 – Number of Proliferating Endothelial Cells (PECs): Normal kidney vs. Antecedent Biopsy (Bx) from CAN grafts

	Normal PEC (n=16)	**CAN Bx PEC (n=18)**
Mean	0.44	4.31
Standard deviation (SD)	0.63	1.81
Standard error of mean (SEM)	0.16	0.45
Range	0 - 2	2 - 8

Mean PEC: Normal vs CAN Bx: $p<0.001$ (Unpaired t-test).

There is a significantly higher number of proliferating endothelial cells in CAN biopsy specimens when compared with normal kidney.

Figure 6.3 – Mean no. of proliferating endothelial cells Normal vs. CAN biopsy



A – Normal

B – CAN Bx

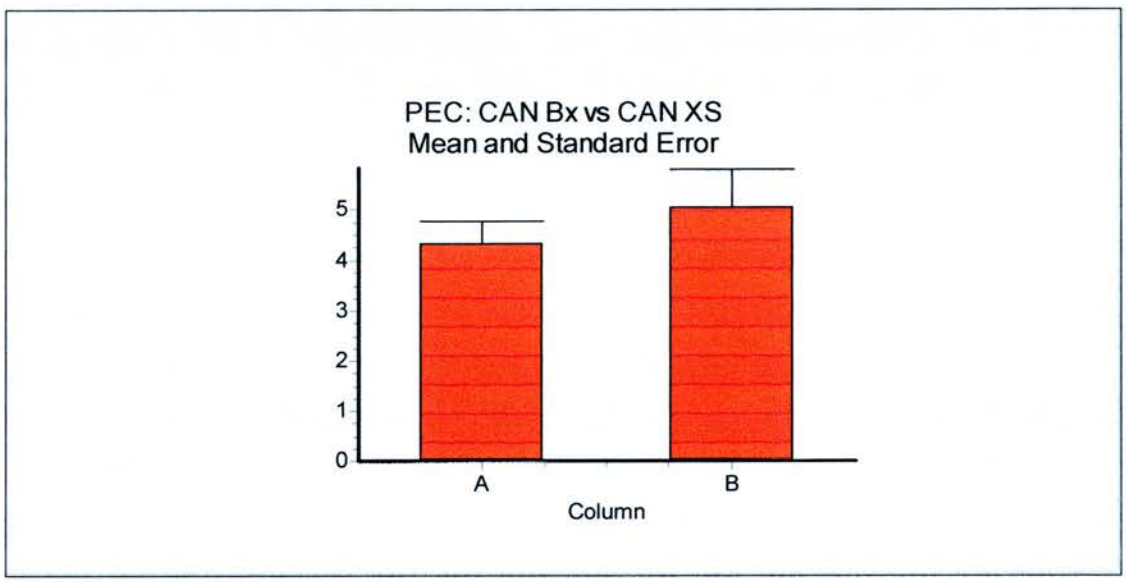
Table 6.4 – Number of Proliferating Endothelial Cells (PECs): Biopsy (Bx) from CAN grafts vs Paired Nephrectomy Specimen (XS)

	CAN Bx PEC (n=18)	**CAN XS PEC (n=18)**
Mean	4.31	5.05
Standard deviation (SD)	1.81	3.17
Standard error of mean (SEM)	0.45	0.74
Range	2 - 8	1 - 15

Mean PEC: CAN Bx vs CAN XS: p=0.471 (Unpaired t-test).

There is no significant difference in the number of proliferating endothelial cells in CAN biopsy specimens when compared with the paired CAN nephrectomy specimen.

Figure 6.4 – Mean no. of proliferating endothelial cells CAN biopsy vs. CAN Nephrectomy



A – CAN Bx

B – CAN XS

Comparison between number of glomerular macrophages in CAN nephrectomy and clinical biopsies

Following staining with the antibody to CD68, the number of glomerular macrophages were counted in several high power (x250) fields. Where possible 20 glomeruli were counted for each section studied.

Sections from 18 clinical biopsies and 29 nephrectomies performed because of CAN were studied as described above. Sections of normal kidney were also studied.

A glomerular macrophage index (GMI) was calculated as;

GMI = Total no. of glomerular macrophages / No. of glomeruli studied per section

Table 6.5 and Figure 6.5 show that CAN nephrectomies have a statistically significant higher GMI than normal kidney.

Howevr, Table 6.6 and Figure 6.6 show that there is a significantly lower GMI in the clinical biopsy specimens when compared with the nephrectomies.

These data do not support or refute a hypothesis that macrophage infiltration is a significant factor in the development of CAN or loss of microvasculature demonstrated in this thesis. As has been mentioned earlier and will be discussed later, there are other factors such as cessation of immunosuppressive therapy that must be taken into account when interpreting these data.

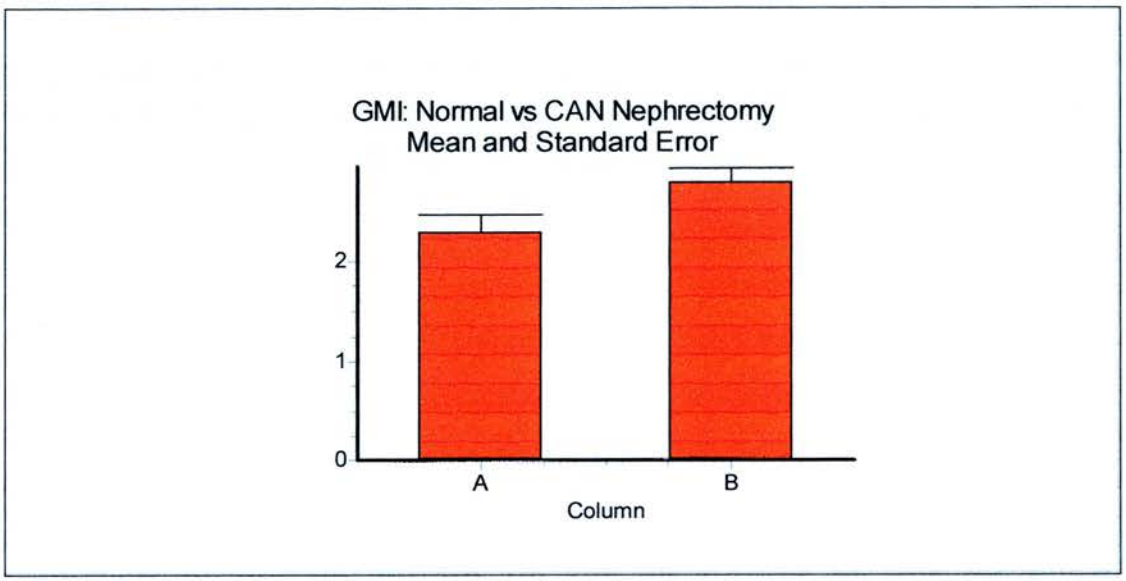
Table 6.5 – Comparison of GMI between Normal Kidney and CAN Nephrectomy

	Normal GMI (n=18)	**CAN XS GMI (n=26)**
Mean	2.29	2.80
Standard deviation (SD)	0.80	0.74
Standard error of mean (SEM)	0.18	0.14
Range	1.25 – 3.90	1.55 – 4.05

Mean GMI: Normal vs CAN nephrectomy: p=0.0368 (unpaired t-test).

There is a significantly higher glomerular macrophage index in CAN nephrectomy specimens when compared with normal kidney.

Figure 6.5 – Mean GMI comparing Normal kidney and CAN nephrectomy



A – Normal

B – CAN Nephrectomy

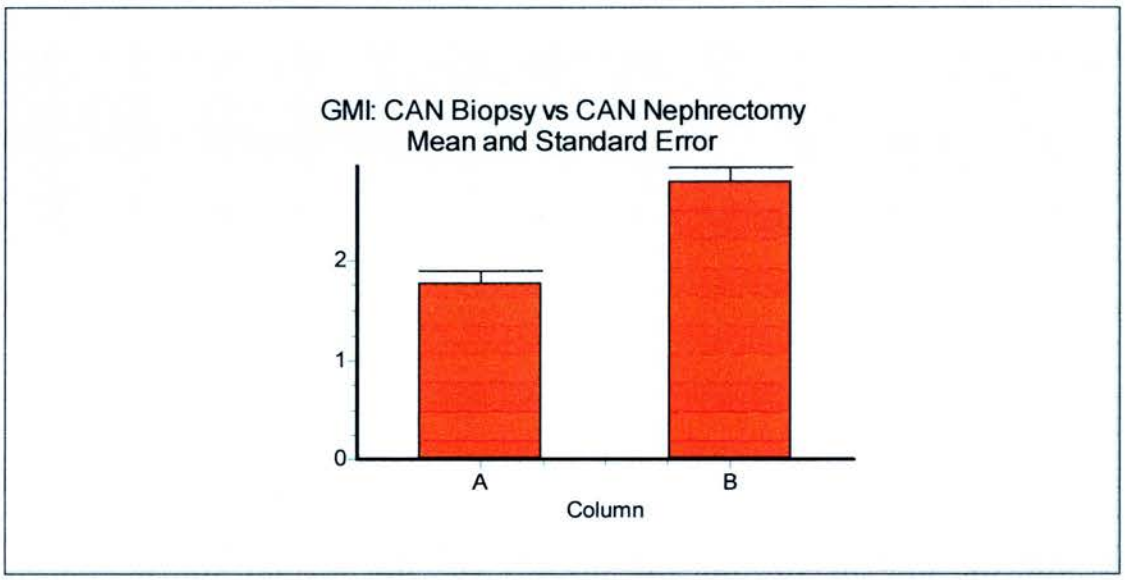
Table 6.6 – Comparison of GMI between CAN Biopsy and CAN Nephrectomy

	CAN Bx GMI	CAN XS GMI
Mean	1.77	2.80
Standard deviation (SD)	0.61	0.74
Standard error of mean (SEM)	0.13	0.14
Range	0.71 – 2.75	1.55 – 4.05

Mean GMI: CAN Biopsy vs CAN nephrectomy: $p<0.0001$ (paired t-test).

There is a significantly higher glomerular macrophage index in CAN nephrectomy specimens when compared with the paired antecedent biopsy.

Figure 6.6 – Mean GMI comparing CAN Biopsy with CAN nephrectomy



A – CAN Bx

B – CAN Nephrectomy

Discussion

Comparison of clinical biopsy with paired nephrectomy specimens has demonstrated the following results:

• MVC is significantly higher in the earlier biopsy specimens but less than that seen in normal kidneys

• proliferating endothelial cells are present in similar numbers throughout the progression of CAN but in significantly higher numbers than in normal kidney

• the number of glomerular macrophages falls in the earlier stages of CAN but then significantly rises as CAN progresses

These data provide further insight into the dynamic process occurring in the vasculature early in the development of CAN and continue to support a hypothesis that angiogenesis is stimulated early following transplantation.

The limitations of this study relate to the tissue used. Nephrectomy specimens were obtained following cessation of immunosuppression which may affect both angiogenesis and macrophage infiltration. The biopsies were performed due to clinical indication e.g. a rise in serum creatinine and the presence of acute rejection or viral infection may be a stimulus for endothelial cell proliferation.

There are two strategies that may be employed to overcome these problems.

i) As described in the Introduction chapter, development of animals of CAN would allow for acquisition of tissue at regular intervals during progression of CAN and in a murine model allow knockout experiments to be conducted e.g. macrophage deplete recipient mice.

ii) The use of protocol biopsy specimens from human allografts.

The former strategy is being developed within our research group but with no tissue yet available for study.

The latter strategy relies on clinicians performing protocol biopsies and this has not been the practice at the Transplant Unit in Edinburgh.

However, following collaboration with the Oxford Transplant Centre where protocol biopsies are performed at day 7 and 28 following transplantation, the following chapter will detail results obtained comparing MVCs in early protocol biopsies.

Chapter 7: Microvessel counts using protocol renal allograft biopsy specimens

Previous results presented have demonstrated that during the progression of CAN there are ongoing attempts at tissue repair as manifested by the presence of proliferating endothelial cells.

Furthermore it has been shown by the use of clinical biopsy specimens that in the earlier stages of CAN there is some preservation of microvasculature compared with end stage disease although there is already evidence of significant microvessel loss in these specimens. However the median timing of these clinical biopsies was 17 months after transplantation (range 1 week to 96 months) and thus unable to provide robust data to examine changes in microvasculature in the first few days and weeks following transplantation.

It is during the initial post-transplant phase that the allograft is subjected to various insults such as:

- host immune response
- ischaemia-reperfusion injury
- nephrotoxic drugs

Processes that occur during this phase may not be immediately manifested clinically and thus clinical biopsies performed because of signs of graft deterioration may not accurately reflect the cellular changes that have occurred.

Debate exists about the merits of performing protocol biopsies and not all clinicians or transplant centres advocate their use. The risks of damaging the transplanted kidney or causing significant morbidity to the patient must be balanced with the

possible benefits of detecting sub-clinical disease. An additional benefit in performing protocol biopsies is to the researcher.

Results

Protocol biopsy specimens from renal transplants were obtained from the Oxford Transplant Centre following ethical approval and written patient consent. Samples were obtained between 1985 and 1996. Follow-up of graft function ranged from 8 to 19 years.

A total of 30 biopsies were studied following immunostaining with antibody to CD31 as described above. Microvessel counts (MVCs) were performed with the investigator blinded to graft outcome.

The 30 patients were then divided into two groups according to graft outcome:

20 patients experienced stable graft function

10 patients developed CAN (confirmed by later clinical biopsy).

Due to restrictions of specimen availability it was not possible to compare MVC in early protocol versus later clinical biopsy in the group where CAN developed.

Timing of protocol biopsies was similar in each group as shown in Table 7.1:

Table 7.1 – Timing of protocol biopsy

	Timing of biopsy (day)
Stable graft	**19 (5 - 90)**
CAN	**20 (7 - 60)**

Median (Range)

These biopsies were therefore taken at an earlier stage than the clinical biopsies studied and presented in the previous chapter and the majority were performed during the first month following transplantation.

Only cortical tissue was available for study and only biopsies with at least three glomeruli were included for study.

As demonstrated in Table 7.2 and Figure 7.1 there was a significant increase in MVC in the biopsies from grafts which went on to develop CAN compared with stable graft group.

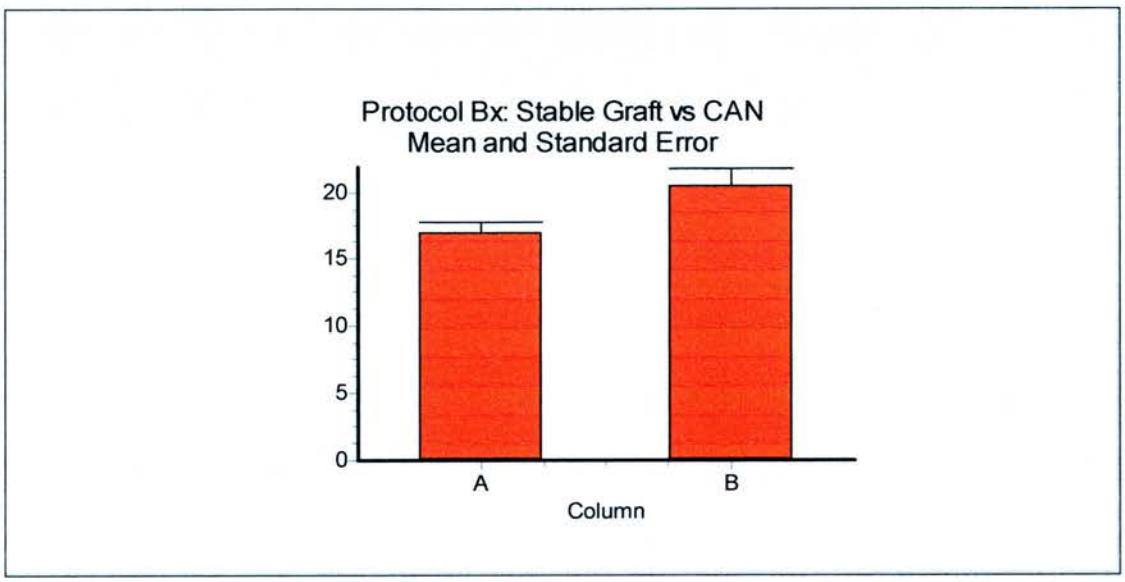
Table 7.2 - MVC in Protocol Biopsies from Patients with Stable Graft Function vs. CAN

	Stable graft	**CAN develops**
Mean	16.93	20.5
Standard deviation (SD)	4.19	3.92
Standard error of mean (SEM)	0.78	1.24
Range	14 - 25	19 - 25

Mean MVC: Stable Grafts vs CAN: p=0.0238

There is a significantly higher MVC in grafts which go on to develop CAN than those in which there is stable graft function.

Figure 7.1 – Mean MVC in Protocol Biopsies from Patients with Stable Graft Function vs. CAN



A – Stable Graft

B – Grafts which develop CAN

Discussion

This study has demonstrated a highly significant expansion of microvasculature during the first month after transplantation in allografts where CAN later develops.

It was not possible with the tissue available to assess for endothelial cell proliferation in this population but it can be postulated that this must be present to explain the higher MVCs described.

These data support our hypothesis that angiogenesis is stimulated early following renal transplantation during the development of CAN and this may provide a novel therapeutic target in the treatment of these patients.

Further studies of mediators of angiogenesis and graft macrophage infiltration in these groups would be of great interest.

Chapter 8: Discussion of changes in vascularity associated with chronic allograft nephropathy

The present study addresses the changes in microvasculature seen in renal allografts with histological changes of CAN (Racusen 1997). The population of end-stage CAN grafts studied had failed at varying times after transplantation resulting in cessation of immunosuppressive therapy, return to renal replacement therapy and graft nephrectomy.

A significant reduction in microvessel count (MVC) in end-stage CAN compared with normal kidney was shown, demonstrating progressive loss of microvasculature with the development of CAN. Previous studies using renal allograft biopsies have shown a significant increase in microvessel density in chronic rejection (Ozdemir et al 2001) and in the expression of the endothelial cell mitogen vascular endothelial growth factor (VEGF) (Pilmore et al 1999). This suggests that earlier in the progression of CAN there may be attempts at expansion of the microvasculature/angiogenesis in response to the hypoxia/insult of transplantation. We tested this hypothesis further by performing MVCs on antecedent renal biopsy specimens from our population of allografts but first sought to validate the use of this technique with renal biopsy specimens.

Assessment of angiogenesis must involve methods which are reliable and reproducible (Vermeulen et al 1996, Vermeulen et al 2002a). This study examined variability in the assessment of angiogenesis in human kidney specimens by comparing observer variation in reporting of microvessel counts and by comparing MVCs of paired specimens of normal kidney cross-section against core biopsy of the same kidney.

A significant correlation between the two observers was demonstrated also at two time points for one observer confirming this method to be reliable. Furthermore a

significant correlation was demonstrated between core biopsies and cross sections of the renal tissue studied, thus validating the use of this method.

The results of these studies were of vital importance and under-pinned the remaining work undertaken which examined MVCs from renal biopsy specimens. Two sets of biopsy samples were studied and have been referred to as clinical and protocol biopsies.

The clinical biopsies were performed because of a clinical indication e.g. rising serum creatinine or significant proteinuria. These biopsy samples were taken early in the development of CAN and paired with nephrectomy samples which demonstrated end stage CAN. This allowed for comparison of microvasculature at differing times during CAN.

The clinical biopsies demonstrated a significantly higher MVC than the corresponding nephrectomy with less distortion of normal microvasculature demonstrated but showed a significant reduction in MVC compared to normal kidney. This confirms both quantitavely and qualitatively that earlier in the development of CAN there was some (but not complete) preservation of normal microvasculature. The present study does not demonstrate the same increase in microvessel density as previous observers (Ozdemir et al 2002) however due to the timing of specimen collection our data did not preclude an early rise in MVC following transplantation. Thus protocol biopsies were used for study.

The protocol biopsies studied were taken during the first month following transplantation and pre-dated the development of CAN. It has been shown that the Banff classification provides a reproducible method for histological assessment of protocol renal allograft biopsies in stable grafts (Gough et al 2002). Such biopsies also have value in detecting subclinical rejection and early CAN and may be used as surrogate efficacy end points in evaluation of therapy in trials aimed to prevent CAN (Gough et al 2002, Seron et al 2000). Despite these benefits, caution has been advocated in their use in clinical decision making. Renal graft biopsy specimens suffer from several limitations (sample size, timing of biopsy, reproducibility and

specificity) and should only be used to complement other clinical criteria (Saad et al 1997, Ponticelli 13 Aug 2002). Furthermore, one study has reported the use of protocol biopsies at 4 and 14 months to monitor the progression of CAN as inaccurate due to the relatively high proportion of misclassified biopsies (Seron et al 2002).

The protocol biopsies studied demonstrated a significantly higher MVC in grafts which later developed CAN than those that did not or where stable graft function continued. This highly significant result supports a hypothesis that angiogenesis is stimulated early in the development of CAN, the clinical significance of which is not yet clear. To further assess the dynamic changes in microvasculature, studies were performed to seek the presence of proliferating endothelial cells in CAN.

Dual immunostaining was performed to identify proliferating endothelial cells (ECs) to assess for the presence of EC repair and angiogenesis. Proliferating ECs were almost absent in normal kidney. There was a significant increase in the number of proliferating ECs in both the CAN nephrectomy specimens and the paired clinical biopsies but no significant difference between these two groups. The presence of proliferating ECs throughout the life of each graft suggests there is a constant stimulus for tissue repair or that this stimulus has not been 'switched off'. These findings support the hypothesis outlined above but that over time, whilst there is persisting evidence of EC proliferation, progressive microvascular loss contributes to the loss of the graft. Recent evidence has confirmed that there are two distinct phases of injury as CAN evolves; an initial phase of early tubulointerstitial damage from ischaemic injury and a later phase of chronic allograft nephropathy characterized by microvascular and glomerular injury (Nankivell et al 2003). Furthermore, glomerulosclerosis (GS) which is characteristic of CAN, has been demonstrated to be a time-dependent response to glomerular injury from early ischaemia, immune-mediated tubular loss and late CNI toxicity (Nankivell et al 2004a).

Immunostaining with antibody to CD31 does not exclusively stain the vascular endothelium but can also stain lymphatics, thus the microvessel counts performed in

this study may have demonstrated lymphatic as well as vascular changes. These findings have since been investigated as part of work following on from this study by immunostaining the same tissues with podoplanin antibody to lymphatic endothelium. This has confirmed that in a subset of the grafts with CAN there is evidence of lymphatic proliferation which is associated with ingress of B lymphocytes and VEGF-C expression (Adair et al 2006, unpublished). As B cells are not known to release VEGF-C their effect may be mediated by macrophages which this study has confirmed to be present in significantly higher numbers in end-stage CAN. It is not clear why only just over half of the grafts studied demonstrated these lymphatic changes whilst others did not.

In the other samples studied with podoplanin there was no evidence of significant lyphangiogenesis confirming that the CD31 counts represent the vascular endothelial staining in these sections.

Future work to establish means of diagnosing, monitoring, preventing and treating CAN must be sought. Ultrasound elasticity imaging has been suggested as a useful tool in measuring mechanical changes related to fibrosis within the transplant kidney which may have efficacy in screening for early CAN (Weitzel et al 2004). The extent of tubulointerstitial fibrosis (TIF) has been shown to correlate significantly with the functional decline of grafts and a new method to quantify TIF has been developed Sirius Red non-polarised strategy) which could be applied to longitudinal studies of CAN (Diaz Encarnacion et al 2004).

As previously stated, a murine model of CAN was not available for study, however advances in the study of CAN are being made in other animal models. More recently it has been demonstrated that application of FTY720 in a rat model ameliorated CAN even at advanced stages and that this effect was achieved by a reduction of graft infiltrating lymphocytes (Wang et al 2004). Translation of these results to humans is not always possible and the majority of current literature regarding treatment of CAN focuses on modulation of immunosuppressive regimens.

The dominant theme in strategies to prevent CAN is to remove or replace long-term calcineurin inhibitors (CNIs) such as cyclosporine or tacrolimus (Lo 2004). Pathological changes of CNI toxicity are virtually universal by 10 years and exacerbated by CAN (Nankivell et al 2004b). The choice of CNI may affect graft outcome although this remains controversial. Whilst some authors report that tacrolimus therapy projects a longer half life (Offermann 2004) with fewer chronic lesions seen on biopsies (Toz et al 2004), these have not been randomised controlled trials. One study has shown preliminary data which confirms that MMF/reduced dose cyclosporine is superior to tacrolimus-for-cyclosporine switch and standard dose cyclosporine for patients with CAN as demonstrated by analysis of reciprocal of creatinine vs. time (ROCT) plots and GFR (Stoves et al 2004). One concern about reduction or omission of CNIs is that a higher incidence of acute rejection will prevail and this is perhaps valid. It is interesting to note however that subclinical acute rejection has not been shown to be associated with a poorer graft outcome (Veronese et al 2004).

Optimisation of blood pressure, dyslipidaemia and the use of angiotensin 2 receptor antagonists have also been advocated as potentially effective concurrent therapies in patients with CAN (Aull 2004).

Expression of the chemokines MCP-1 and RANTES is significantly upregulated in acute rejection compared with CAN and this correlated with momocyte/macrophage infiltration (Ruster et al 2004). Since chemokines are not only involved in inflammation but also tissue regeneration this could have impact on the development of CAN.

The protein-crosslinking enzyme, tissue transgluaminase (tTg) has been implicated in renal fibrosis. tTg, and its crosslink product epsilon-(gamma-glutamyl) lysine, correlated with the initiation and progression of scarring on sequential biopsies from renal allograft recipients who experienced CAN (Johnson et al 2004). Elevated tTg may therefore be an early predictor of the development of CAN and tTg manipulation to prevent fibrosis may offer an alternate therapeutic strategy.

This thesis sought to examine a role for the therapeutic and prognostic implications of changes in microvasculature during CAN. Angiogenesis has been widely studied in cancer and inflammatory diseases and regulation of angiogenesis is governed by a balance between pro- and anti-angiogenic mediators (Hanahan). Up-regulation of VEGF has been shown in chronic renal allograft rejection (Pilmore 1999) and was associated with the development of fibrosis. Recent work investigating the role of the microvascular endothelium in progressive renal disease has demonstrated an early proliferative response of the peritubular endothelium to injury (Kang 2001). This proliferation was not sustained, with progressive capillary loss and interstitial fibrosis. These changes were associated with alterations in the balance of angiogenic mediators, specifically VEGF and the inhibitor of angiogenesis thrombospndin-1 (Kang 2001). In vitro study of ECs challenged by allogenic lymphmonocytes demonstrated that smooth muscle cells co-cultured with ECs can be stimulated to trans-differentiate from contractile to synthetic cells which further supports a role for the ECs in the progression of the fibrosis seen in CAN (Amore 2001). It seems likely that the changes in vascularity and EC proliferation we have described will depend on the balance of angiogenic mediators within each graft and that VEGF may have an important role. Defining changes in vascularity associated with CAN and the mechanisms involved may allow for the development of novel diagnostic and therapeutic strategies to identify and protect grafts.

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Appendices

Appendix to Materials and Methods

Materials:

Preparation of all specimens:

Formal saline	ADAMS HEALHCARE 416479
Mounting cassettes	BDH 404/0519/02
Superfrost microscope slides	BDH 406/0179/00
Coverslips	

Reagents for dewaxing of sections:

Xylene		
Abs alcohol		
Isopropanol	MERCK	Lot number: 102246L

Staining for histological sections:

Haematoxylin

Eosin

Reagents for immunohistochemistry:

Tris buffered saline (TBS):

TRIZMA base.	SIGMA T – 1503	Lot number: 16H5738
Sodium chloride	FISHER S/3120/53	Lot number: 9741598 227

0.9% solution prepared in distilled water, by adding 9g sodium chloride to 1 litre distilled water.

Hydrochloric acid FISHER H/1000/PB17

To make up TBS: 60.55g Trizma base was added to 1 litre distilled water and pH optimized to 7.6 by the addition of concentrated hydrochloric acid.

0.05M TBS was used for staining; to achieve this concentration, 100mls TBS was added to 900ml 0.9% sodium chloride.

Phosphate buffered saline

100 tablets dissolved in 10 litres distilled water.

Hydrogen peroxide

SIGMA H-1009

Blocking reagents:

Avidin biotin blocking kit	VECTOR	Lot number SP-2001
Serum free protein block	DAKO X0909	

Reagents for antigen retrieval

Protease Type XXIV	SIGMA P-8038	Lot number: 86H0561
Citric acid	SIGMA C-7129	Lot number: 96H0355
Antigen unmasking solution	VECTOR	

Primary antibodies

Mouse anti-human CD31 monoclonal antibody. Clone JC/70A

DAKO M0823 Lot number: 016 (201)

Rat anti-mouse CD31 monoclonal antibody (MEC 13.3)

PHARMIGEN 01951D Lot number: M022293

Rabbit anti-human VEGF polyclonal antibody.

SANTA CRUZ BIOTECHNOLOGY Code sc:507

Mouse anti-human MIB-1 monoclonal antibody

DAKO

Mouse anti-human CD68 monoclonal antibody

DAKO

Secondary antibodies:

Rabbit anti-mouse immunoglobulin

Rabbit anti-mouse immunoglobulin (biotin)

Rabbit anti-rat immunoglobulin (APAAP)

Tertiary systems:

APAAP, mouse, monoclonal	DAKO D0651	Lot number: 106 (101)
APAAP, rat, monoclonal	DAKO D0488	Lot number: 046 (102)
ABC reagent	VECTOR	

Visualisation systems:

New Fuschin Substrate system	DAKO K698

Diaminobenzidine (DAB)

Mounting media

Xylene Substitute Mountant	SHANDON code: 1900233

Methods:

General Immunohistochemistry Protocol using ABC/DAB for CD68 and MIB-1 Antibodies

1. Dewax sections in 2 changes of xylene (5 mins each) and rehydrate through graded alcohols: absolute alcohol 5 mins; 70% alcohol 5 mins; IMS 5 mins; to distilled water.
2. Block endogenous peroxidase in 3% hydrogen peroxide for 10 mins (Sigma code: H-1009: 30mls of this in 270mls distilled water).
3. Perform antigen retrieval as required (see footnote).
4. Wash in distilled water @ room temperature.
5. Transfer sections to sequenza (or equivalent) and wash through with TBS.
6. Using Vector blocking kit (code: SP-2001) apply 3 drops of Avidin per section and leave for 10-15 mins. Wash briefly with TBS.
7. Apply3 drops of Biotin from same kit and leave for 10-15 mins. Wash with TBS.
8. Apply 3 drops of Dako Protein Block (code: X0909) and leave for 15 mins.
9. Apply 100µl of primary antibody per section @ appropriate dilution. Leave for 1 or 2 hours or overnight (depending on antibody). Wash in TBS.
10. Apply secondary antibody @ 1:300 at appropriate dilution. Leave for 30 mins. Wash in TBS.
11. Apply 3 drops of Vector ABC Vectastain reagent (code: PK-7100) per section. Leave for 30 mins. Wash in TBS. Wash through with distilled water.

12. Mark coverplates with waterproof pen (can be used 6 times each before disposal).
13. Place sections on a staining rack over sink and cover with distilled water to prevent them drying out.
14. Make up DAB using the Dako DAB kit (code: K3468). (Take suitable precautions when using DAB). Wipe away water carefully around sections and apply DAB. Leave on for 10 minutes. Dispose of surplus DAB carefully in fume hood sink. Place sections in a staining rack and rinse well in tap water.
15. Intensify in Copper Enhancement solution (6.66g cupric sulphate and 11.88g sodium chloride dissolved in 1000mls deionised water) for 5 mins and wash again in tap water.
16. Counterstain with haematoxylin (1-2 min), differentiate in acid alcohol (dip in a few times), blue in ammonia water (1 min). Check under microscope and adjust counterstain as necessary.
17. Dehydrate through alcohols (IMS 5mins; 70% alcohol 5 mins; absolute alcohol 5 mins), Clear through 2 changes of xylene (5 mins each) and mount in DPX.

Footnote: Microwave Antigen Retrieval: Place sections in a plastic rack inside a plastic Tupperware filled with Vector antigen retrieval solution and microwave for 3 blasts of 5 minutes checking for evaporation between each blast. Leave to cool for 20 minutes before transferring quickly to distilled water, ensuring sections do not dry out.

General Immunohistochemistry Protocol using APAAP/New Fuchsin for CD31 Antibodies

1. Dewax sections in 2 changes of xylene (5 mins each) and rehydrate through graded alcohols: absolute alcohol 5 mins; 70% alcohol 5 mins; IMS 5 mins; to distilled water.
2. Perform protease antigen retrieval (see footnote).
3. Transfer sections to sequenza or equivalent and wash through with TBS (pH 7.6).
4. Apply 3 drops of Dako Protein Block (serum free) (code: X0909) and leave for 30 mins.
5. Apply primary CD31 antibody @ 1:20 dilution. Leave for 1 hour at room temperature. Wash in TBS.
6. Apply secondary antibody (rabbit anti-mouse immunoglobulins) @ 1:50 dilution. Leave for 30 mins. Wash in TBS.
7. Apply APAAP reagent @ 1:100 dilution. Leave for 30 mins. Wash in TBS.
8. Repeat steps 6 and 7 this time incubating each for only 10 minutes, to enhance the signal. Mark coverplates and wash through with distilled water.
9. Place sections on a staining rack over sink and cover with distilled water to prevent them drying out.
10. Make up New Fuchsin Substrate using kit (Dako code: K0698). Wipe round sections and apply new fuchsin. Leave for 20 minutes.
11. Wash in distilled water.

12. Counterstain with haematoxylin, differentiate in acid alcohol, blue in ammonia water. Check under microscope and adjust counterstain as necessary.
13. Dehydrate through alcohols (IMS 5mins; 70% alcohol 5 mins; absolute alcohol 5 mins), clear through isopropanol (xylene dissolves new fuchsin) and mount in Xylene Substitute Mountant (Shandon code: 1900233) or other aqueous mountant.

Protease antigen retrieval – make up fresh for each use. Incubate sections in warm distilled water heated to 37°C for 10 minutes. Make up protease (Sigma code: P-8038) in the pre-warmed PBS (12.5mg/100ml PBS) and incubate slides in this for exactly 20 minutes @ 37°C. Timing is crucial.

Immunohistochemistry Protocol using ABC and APAAP for MIB-1/CD31 Dual stain

1. (Pre-heat distilled water and PBS to 37°C if using protease for antigen retrieval.)
2. Dewax sections in 2 changes of xylene (5 mins each) and rehydrate through graded alcohols: absolute alcohol 5 mins; 70% alcohol 5 mins; IMS 5 mins; to distilled water.
3. Block endogenous peroxidase in 3% hydrogen peroxide for 10 mins (Sigma code: H-1009: 30mls of this in 270mls distilled water).
4. Perform microwave antigen retrieval.
5. Wash in distilled water @ room temperature.
6. Transfer sections to sequenza (or equivalent) and wash through with TBS.
7. Using Vector blocking kit (code: SP-2001) apply 3 drops of Avidin per section and leave for 10-15 mins. Wash briefly with TBS.
8. Apply3 drops of Biotin from same kit and leave for 10-15 mins. Wash with TBS.
9. Apply 3 drops of Dako Protein Block (code: X0909) and leave for 15 mins.
10. Apply 100µl of primary antibody per section @ appropriate dilution. Leave for 1 or 2 hours or overnight (depending on antibody). Wash in TBS.
11. Apply secondary antibody @ 1:300 at appropriate dilution. Leave for 30 mins. Wash in TBS.
12. Apply 3 drops of Vector ABC Vectastain reagent (code: PK-7100) per section. Leave for 30 mins. Wash in TBS. Wash through with distilled water.

13. Mark coverplates with waterproof pen (can be used 6 times each before disposal).
14. Place sections on a staining rack over sink and cover with distilled water to prevent them drying out.
15. Make up DAB using the Dako DAB kit (code: K3468). (Take suitable precautions when using DAB). Wipe away water carefully around sections and apply DAB. Leave on for 10 minutes. Dispose of surplus DAB carefully in fume hood sink. Place sections in a staining rack and rinse well in tap water.
16. Intensify in Copper Enhancement solution (6.66g cupric sulphate and 11.88g sodium chloride dissolved in 1000mls deionised water) for 5 mins and wash again in tap water (optional).
17. Transfer sections to sequenza or equivalent and wash through with TBS (pH 7.6).
18. Apply 3 drops of Dako Protein Block (serum free) (code: X0909) and leave for 30 mins.
19. Apply primary antibody @ titrated dilution. Leave overnight. Wash in TBS.
20. Apply secondary antibody @ titrated dilution. Leave for 30 mins. Wash in TBS.
21. Apply appropriate APAAP reagent @ titrated dilution. Leave for 30 mins. Wash in TBS.
22. Repeat steps 20 and 21 this time incubating each for only 10 minutes, to enhance the signal. Mark coverplates and wash through with distilled water.

23. Place sections on a staining rack over sink and cover with distilled water to prevent them drying out.
24. Make up New Fuchsin Substrate using kit (Dako code: K0698). Wipe round sections and apply new fuchsin. Leave for 20 minutes.
25. Wash in distilled water.
26. Counterstain with haematoxylin, differentiate in acid alcohol, blue in ammonia water. Check under microscope and adjust counterstain as necessary.
27. Dehydrate through alcohols (IMS 5mins; 70% alcohol 5 mins; absolute alcohol 5 mins), clear through isopropanol (xylene dissolves new fuchsin) and mount in Xylene Substitute Mountant (Shandon code: 1900233) or other aqueous mountant.

1. Parathyroid MVC and VEGF data

Parathyroid MVC – Normal parathyroid

Pathology no.	Count 1	Count 2	Count 3	Total MVC	Tissue diagnosis
23029/94	4	4	2	10	normal
11524/96	2	2	2	6	normal
1509/96 II	2	1	1	4	normal
1530/96 I	4	3	3	10	normal
11509/96 II	3	6	2	11	normal
22101/98 II	6	5	8	19	normal
19452/98 II	8	4	3	15	normal
20923/96 III	4	2	6	12	normal
1634/97 II	3	5	4	12	normal
25101/98 I	3	3	5	11	normal
17799/98 I	9	4	4	17	normal
7857/93 III	11	18	14	43	normal
17623/97 II	3	4	4	11	normal
16558/96 II	5	5	3	13	normal
3455/99 IV	4	4	3	11	normal
16105/98 II	4	2	3	9	normal
4019/98 II	1	1	1	3	normal
10024/98 III	9	10	6	25	normal
10980/98 I	4	3	2	9	normal
23283/97 II	2	1	2	5	normal
8720/98 III	5	7	9	21	normal
10191/98 I	5	11	8	24	normal
17259/97 II	2	3	3	8	normal
17259/97 I	3	6	3	12	normal
23886/90 II	7	10	6	23	normal
3896/90 III	3	2	2	7	normal
8506/00 VI	5	3	4	11	normal
17565/90 III	3	4	3	10	normal
22481/98 III	3	4	3	10	normal
20923/96 IV	9	5	7	21	normal
23029/94 III	6	7	4	17	normal
20182/97 I	8	6	4	18	normal
4809/95 I	3	4	4	11	normal
20684/91 I	4	7	4	15	normal
20282/91 III					
283/91 I					
19028/91 II					
13580/90 II					
17799/98 II					
24157/91 II					
2519/92 II					
23581/91 II					
13311/95 II					
9826/90 III					
18293/93 II					

Parathyroid MVC – Parathyroid adenoma

Pathology no.	Count 1	Count 2	Count 3	Total MVC	Tissue diagnosis
21705/92	9	10	23	42	adenoma
1530/96	23	9	10	42	adenoma
11509/96	17	17	10	44	adenoma
25806/94	17	11	5	33	adenoma
25381/91	19	11	4	34	adenoma
24157/91	13	11	7	31	adenoma
18293/93	10	8	7	25	adenoma
2519/92	10	8	14	32	adenoma
1634/97	10	11	7	28	adenoma
25300/98	21	11	18	50	adenoma
20236/97	12	9	10	31	adenoma
13580/90	9	8	12	29	adenoma
20764/90	5	6	3	14	adenoma
21374/90	3	3	4	10	adenoma
21693/90	10	11	6	27	adenoma
7045/90	5	3	4	12	adenoma
1840/91	12	14	15	41	adenoma
2608/91	6	5	7	18	adenoma
283/91	25	9	7	41	adenoma
2395/91	11	18	10	39	adenoma
12274/91	18	20	9	47	adenoma
20282/91	13	6	6	25	adenoma
19361/91	8	9	9	26	adenoma
19028/91	10	7	11	28	adenoma
17589/90	6	3	4	13	adenoma
25101/97	7	10	9	26	adenoma
22101/98	10	11	8	29	adenoma
19452/98	10	9	8	27	adenoma
16558/96	14	12	10	36	adenoma
1331/95	8	10	7	25	adenoma
20303/95	13	25	16	54	adenoma
1509/96	9	24	14	47	adenoma
23029/94 I	14	11	10	35	adenoma
7857/93	11	8	9	28	adenoma
3956/98	8	11	16	35	adenoma
25632/96	12	9	8	29	adenoma
656/98	21	17	14	52	adenoma
9186/98	10	14	10	34	adenoma
17259/97	4	5	3	12	adenoma
23283/97	7	6	7	20	adenoma
16105/98	9	10	13	32	adenoma
4019/98	11	11	9	31	adenoma
10980/98 II	9	14	10	33	adenoma
9826/90	12	7	10	29	adenoma

Parathyroid MVC – Parathyroid hyperplasia

Pathology no.	Count 1	Count 2	Count 3	Total MVC	Tissue diagnosis
13449/96	5	10	12	27	hyperplasia
13481/96	8	6	5	19	hyperplasia
7636/97	10	8	10	28	hyperplasia
12252/96	8	8	8	24	hyperplasia
12887/96	17	7	10	39	hyperplasia
8018/96	8	7	7	23	hyperplasia
18043/95	12	11	5	28	hyperplasia
10867/98	13	8	8	29	hyperplasia
6895/92	4	2	2	8	hyperplasia
25902/95	6	6	4	16	hyperplasia
8716/97	6	11	8	25	hyperplasia
12247/96	3	5	4	12	hyperplasia
4527/97	4	4	4	12	hyperplasia
14883/99	7	14	11	32	hyperplasia
4527/97	9	7	6	22	hyperplasia
3455/99	10	7	8	25	hyperplasia
8497/00	9	6	8	23	hyperplasia
14883/99	9	14	10	33	hyperplasia
14737/99	19	12	8	39	hyperplasia
9298/98	9	10	11	30	hyperplasia
7190/98	19	15	10	44	hyperplasia
26254/99	9	9	11	29	hyperplasia
14883/99	7	10	7	24	hyperplasia
1307/97	10	6	7	23	hyperplasia
8174/98	8	9	10	27	hyperplasia
14883/99 I	4	3	5	12	hyperplasia
4651/97	5	3	4	12	hyperplasia
3560/97	7	3	4	14	hyperplasia

Parathyroid VEGF scores – Normal parathyroid

Pathology no.	Intensity of staining	Proportion of cells staining	Tissue Diagnosis
24157/91 II	2	1	Normal
2519/92 II	2	1	Normal
23581/91 II	2	1	Normal
1509/96 II	3	3	Normal
20303/95 II	1	1	Normal
2815/92 II	2	2	Normal
21705/92 II	1	3	Normal
7857/93 III	1	1	Normal
18293/93 II	2	1	Normal
23029/94 II	2	2	Normal
13311/95 II	2	2	Normal
13449/96 I	1	1	Normal
25806/94 II	2	1	Normal
20684/91 III	1	1	Normal
20282/91 III	1	1	Normal
16558/96 II	1	1	Normal
20923/96 I	2	1	Normal
19028/91 II	3	1	Normal
17259/97 III	3	1	Normal
2608/91 II	1	1	Normal
19361/91 II	1	1	Normal
17623/97 II	2	1	Normal
7045/90 II	1	1	Normal
11509/96 II	2	1	Normal
23886/90 III	1	1	Normal
17565/90 I	1	1	Normal
11524/96 II	1	1	Normal
20923/96 III	3	3	Normal
1634/97 II	2	1	Normal
16105/98 II	1	1	Normal
13580/90 II	3	1	Normal
17799/98 II	2	3	Normal
17799/98 II	1	1	Normal
21693/90 II	2	3	Normal
19452/98 II	2	2	Normal
9826/90 III	1	1	Normal
22101/98 II	2	3	Normal
283/91 I	3	2	Normal

Parathyroid VEGF scores – Parathyroid adenoma

Pathology no.	Intensity of staining	Proportion of cells staining	Tissue Diagnosis
22101/98 I	3	1	Adenoma
283/91 II	1	3	Adenoma
9826/90 II	2	1	Adenoma
19452/98 I	1	1	Adenoma
21693/90 I	1	1	Adenoma
17799/98 I	3	1	Adenoma
13580/90 I	3	1	Adenoma
11524/96 I	1	1	Adenoma
23886/90 I	2	2	Adenoma
17623/97 I	1	1	Adenoma
17259/97 I	1	2	Adenoma
1634/97 II	1	3	Adenoma
17589/90 I	0	0	Adenoma
20764/90 I	1	1	Adenoma
21374/90 I	1	1	Adenoma
7045/90 I	1	1	Adenoma
16558/96 I	1	1	Adenoma
12274/91 I	1	1	Adenoma
2608/91 I	1	1	Adenoma
11509/96 I	1	2	Adenoma
7857/93 IV	1	1	Adenoma
13311/95 I	2	1	Adenoma
2395/91 I	3	1	Adenoma
20282/91 II	2	1	Adenoma
20684/91 II	0	0	Adenoma
24157/91 I	2	1	Adenoma
18840/91 II	0	0	Adenoma
18293/93 I	1	1	Adenoma
19361/91 II	0	0	Adenoma
19028/91 I	1	1	Adenoma
1509/96 I	1	1	Adenoma
2519/92 I	0	0	Adenoma
21705/92 I	1	1	Adenoma
23029/94 I	2	2	Adenoma
23581/91 I	1	1	Adenoma
20303/95 I	2	1	Adenoma
25806/94 I	3	1	Adenoma
2815/92 III	1	1	Adenoma

Parathyroid VEGF scores – Parathyroid hyperplasia

Pathology no.	Intensity of staining	Proportion of cells staining	Tissue Diagnosis
8018/96	3	1	Hyperplasia
12247/96 I	3	1	Hyperplasia
12252/96 II	1	1	Hyperplasia
12887/96 I	1	1	Hyperplasia
4527/97 I	1	1	Hyperplasia
7636/97 III	1	1	Hyperplasia
8716/97 I	1	1	Hyperplasia
23571/90 I	2	1	Hyperplasia
13449/96 III	1	3	Hyperplasia
25902/95 I	1	1	Hyperplasia
3560/97 III	3	1	Hyperplasia
6895/92 I	1	1	Hyperplasia
18043/95 I	1	1	Hyperplasia

2. Validation of microvessel counts in renal biopsy specimens

Normal kidney MVC comparing cross section (XS) and biopsy (Bx) from same kidney - Cortex

Specimen	XS glomeruli	XS count	Bx glomeruli	Bx count
K1	4	25	4	25
K2	3	22	2	19
K3	6	20	5	20
K4	3	30	3	28
K5	5	27	3	27
K6	3	31	n/a	n/a
K7	4	29	4	28
K8	3	20	n/a	n/a
K9	4	32	3	21
K10	4	23	3	22
K11	5	21	4	20
K12	3	17	4	22
K13	4	20	3	20
K14	4	14	n/a	n/a
K15	4	25	n/a	n/a
K16	3	22	3	21
K17	3	20	3	20
K18	4	22	6	22
K19	7	24	3	20
K20	n/a	n/a	n/a	n/a
K21	3	21	n/a	n/a
K22	5	17	n/a	n/a
K23	4	23	3	22
K24	4	18	3	18
K25	3	12	3	11
K26	3	21	4	16
K27	5	20	2	20

Normal kidney MVC comparing cross section (XS) and biopsy (Bx) from same kidney - Medulla

Specimen	XS count	Bx count
K1	29	23
K2	30	27
K3	18	n/a
K4	23	22
K5	20	n/a
K6	17	18
K7	22	22
K8	21	22
K9	22	25
K10	18	16
K11	20	20
K12	17	n/a
K13	13	19
K14	20	20
K15	27	26
K16	25	24
K17	23	n/a
K18	21	20
K19	n/a	n/a
K20	n/a	n/a
K21	n/a	n/a
K22	n/a	n/a
K23	16	16
K24	16	15
K25	n/a	n/a
K26	n/a	n/a
K27	22	20

3. Comparing microvessel counts in renal tumours and normal kidney

Tumour MVC comparing cross section (XS) and biopsy (Bx) from same kidney

Specimen	Tumour XS	Tumour Bx
K3	37	26
K4	n/a	n/a
K5	30	22
K6	30	n/a
K7	23	22
K8	n/a	n/a
K9	22	20
K10	21	23
K11	20	22
K12	28	31
K13	24	28
K14	29	30
K15	35	29
K16	33	38
K17	26	29
K18	16	16
K19	n/a	n/a
K20	40	37
K21	20	24
K22	21	15
K23	n/a	n/a
K24	23	27
K25	28	27
K26	22	20
K27	29	27

Normal vs. Tumour MVC from the same kidney – cross section

Specimen	Normal cortex	Normal medulla	Tumour XS
K2	22	30	n/a
K3	20	18	37
K4	30	23	n/a
K5	27	20	30
K6	31	17	30
K7	29	22	23
K8	20	21	n/a
K9	32	22	22
K10	23	18	21
K11	21	20	20
K12	17	17	28
K13	20	13	24
K14	14	20	29
K15	25	27	35
K16	22	25	33
K17	20	23	26
K18	22	21	23
K19	24	n/a	n/a
K20	n/a	n/a	40
K21	21	n/a	20
K22	17	n/a	21
K23	23	16	n/a
K24	18	16	23
K25	12	n/a	28
K26	21	n/a	22
K27	20	22	29

Normal vs. Tumour MVC from the same kidney – biopsy

Specimen	Tumour BX	Normal Cortex Bx	Normal Medulla Bx
K2	n/a	19	27
K3	26	20	n/a
K4	n/a	28	22
K5	22	27	n/a
K6	n/a	n/a	18
K7	22	28	22
K8	n/a	n/a	22
K9	20	21	25
K10	23	22	16
K11	22	20	20
K12	31	22	n/a
K13	28	20	19
K14	30	n/a	20
K15	29	n/a	26
K16	38	21	24
K17	29	20	n/a
K18	24	22	20
K19	n/a	20	n/a
K20	37	n/a	n/a
K21	24	n/a	n/a
K22	15	n/a	n/a
K23	n/a	22	16
K24	27	18	15
K25	27	11	n/a
K26	20	16	n/a
K27	27	20	20

Median MVC for both Cross Section and Biopsies from Normal Kidney and Tumour

	Normal Cortex	Normal Medulla	Tumour
Cross Sections	21	20.5	26
Biopsies	20	20	26.5

Student t test $p<0.05$

4. Reproducibility of microvessel counts in human kidney specimens

Intra-observer variation. Counts for DM – cortex

Specimen	DM count 1	DM count 2
K1 XS	24	27
K2 XS	22	20
K3 XS	20	21
K4 XS	30	26
K5 XS	27	27
K6 XS	31	33
K7 XS	29	26
K8 XS	20	21
K9 XS	31	28
K10 XS	22	22
K11 XS	21	23
K12 XS	17	24
K13 XS	19	18
K14 XS	15	18
K15 XS	25	23
K16 XS	23	23
K17 XS	19	20
K18 XS	22	20
K19 XS	23	25
K21 XS	23	25
K22 XS	17	17
K23 XS	21	19
K24 XS	18	12
K25 XS	12	12
K26 XS	20	22
K27 XS	20	18
K1 Bx	24	21
K2 Bx	18	23
K3 Bx	20	19
K4 Bx	26	27
K5 Bx	27	25
K7 Bx	28	27
K9 Bx	21	20
K10 Bx	22	22
K11 Bx	20	21
K12 Bx	22	20
K13 Bx	19	20
K15 Bx	21	21
K16 Bx	21	19
K17 Bx	20	19
K18 Bx	21	22
K19 Bx	20	22
K23 Bx	22	21
K24 Bx	19	14
K25 Bx	11	12
K26 Bx	16	15
K27 Bx	19	15

Intra-observer variation. Counts for DM – medulla

Specimen	DM Medullary count 1	DM Medullary count 2
K1 XS	28	27
K2 XS	30	25
K3 XS	18	20
K4 XS	23	23
K5 XS	20	18
K6 XS	16	20
K7 XS	22	21
K8 XS	20	19
K9 XS	20	21
K10 XS	18	21
K11 XS	20	21
K12 XS	17	19
K13 XS	12	14
K14 XS	19	17
K15 XS	27	27
K16 XS	24	22
K17 XS	22	21
K18 XS	20	21
K23 XS	15	16
K24 XS	15	16
K27 XS	22	21
K1 Bx	28	27
K4 Bx	21	24
K6 Bx	19	21
K7 Bx	23	21
K8 Bx	21	21
K9 Bx	24	20
K10 Bx	15	16
K11 Bx	20	20
K13 Bx	18	18
K14 Bx	20	21
K15 Bx	26	24
K16 Bx	23	24
K18 Bx	20	21
K23 Bx	14	12
K24 Bx	15	14
K27 Bx	20	18

Inter-observer Variation. DM and LM counts – cortex

Specimen	DM count	LM count
K1	24	25
K2	22	21
K3	20	20
K4	30	30
K5	27	27
K6	31	31
K7	29	30
K8	20	20
K9	31	33
K10	22	23
K11	21	20
K12	17	17
K13	19	19
K14	15	12
K15	25	25
K16	23	22
K17	19	21
K18	22	22
K19	23	25
K21	23	20
K22	17	17
K23	21	23
K24	18	17
K25	12	12
K26	20	21
K27	20	20

Inter-observer Variation. DM and LM counts – Medulla

Specimen	DM count	LM count
K1	28	29
K2	30	30
K3	18	18
K4	23	22
K5	20	20
K6	16	17
K7	22	23
K8	20	20
K9	20	22
K10	18	20
K11	20	20
K12	17	17
K13	12	13
K14	19	21
K15	27	27
K16	24	25
K17	22	24
K18	20	21
K23	15	16
K24	15	16
K27	22	22

4. Changes in vascularity associated with chronic allograft nephropathy

MVC comparing CAN Nephrectomy with Normal Kidney - Cortex

Specimen number	CAN nephrectomy MVC		Specimen	Normal kidney MVC	Specimen	Normal kidney MVC cont.
UB 018110R/93	14		8977	21	K1	25
UB 011928M/00	14		9231	n/a	K2	22
UB 020768A/95	18		9493	22	K3	20
UB 018837C/94	12		9644	23	K4	30
UB 017742C/02	13		10333	23	K5	27
UB 003798M/99	16		11073	27	K6	31
UB 019010F/00	18		11406	27	K7	29
UB 005718S/02	16		11940	25	K8	20
UB 016885J/02	n/a		11964	22	K9	32
UB 006779/90	17		12242i	29	K10	23
UB 011293B/01	13		12242j	20	K11	21
UB 017246P/00	18		12851	20	K12	17
UB 026006T/02	22		13394	22	K13	20
UB 023656F/98	13		462	22	K14	14
UB 017275K/96	17		897	26	K15	25
UB 023572K/94	15		899	22	K16	22
UB 018005S/99	11		1426	17	K17	20
UB 021907/95	16		2005	21	K18	22
UB 020561H/91	14		2226	30	K19	24
UB 018961G/97	12		2526	20	K20	n/a
UB 014924S/00	14		4152	22	K21	21
UB 020972L/90	13		4381	22	K22	17
UB 024483B/00	15		4502	26	K23	23
UB 001909C/98	14		5196	24	K24	18
UB 012398L/98	12		5192	21	K25	12
UB 002921C/96	14		6552	25	K26	21
UB 012066A/01	15		7439	19	K27	20
UB 003081/97	12		7812	19		
UB 006358/97	18		7945	20		
UB 008353/98	21		8880	21		
UB 019767/99	12		9325	28		
UB 005247/00	16		9943	21		
UB 023079/01	15		10083	23		
UB 023957/01	15		10521	21		
UB 025179/01	18		10879	20		

MVC comparing CAN Nephrectomy with Normal Kidney – Medulla

Specimen number	CAN nephrectomy		Specimen	Normal kidney	Specimen	Normal kidney cont.
UB 018110R/93	n/a		8977	19	K1	29
UB 011928M/00	14		9231	n/a	K2	30
UB 020768A/95	14		9493	20	K3	18
UB 018837C/94	n/a		9644	n/a	K4	23
UB 017742C/02	15		10333	25	K5	20
UB 003798M/99	18		11073	24	K6	17
UB 019010F/00	15		11406	19	K7	22
UB 005718S/02	17		11940	23	K8	21
UB 016885J/02	16		11964	n/a	K9	22
UB 006779/90	15		12242i	n/a	K10	18
UB 011293B/01	14		12242j	n/a	K11	20
UB 017246P/00	14		12851	n/a	K12	17
UB 026006T/02	23		13394	25	K13	13
UB 023656F/98	14		462	22	K14	20
UB 017275K/96	14		897	25	K15	27
UB 023572K/94	11		899	21	K16	25
UB 018005S/99	22		1426	n/a	K17	23
UB 021907/95	13		2005	n/a	K18	21
UB 020561H/91	15		2226	29	K19	n/a
UB 018961G/97	14		2526	18	K20	n/a
UB 014924S/00	16		4152	24	K21	n/a
UB 020972L/90	n/a		4381	21	K22	n/a
UB 024483B/00	17		4502	25	K23	16
UB 001909C/98	18		5196	26	K24	16
UB 012398L/98	18		5192	n/a	K25	n/a
UB 002921C/96	19		6552	n/a	K26	n/a
UB 012066A/01	12		7439	18	K27	22
UB 003081/97	11		7812	18		
UB 006358/97	13		7945	19		
UB 008353/98	17		8880	21		
UB 019767/99	14		9325	26		
UB 005247/00	12		9943	17		
UB 023079/01	16		10083	24		
UB 023957/01	13		10521	22		
UB 025179/01	11		10879	17		

No. of Proliferating Endothelial Cells (ECs) and Proliferation Index – Normal Kidney

Specimen	No. proliferating ECs	Total no. proliferating cells	Proliferation Index
K1	0	13	0
K2	0	11	0
K3	0	6	0
K4	1	18	0.055555556
K5	1	9	0.111111111
K6	0	16	0
K7	0	6	0
K8	0	7	0
K9	0	14	0
K10	0	13	0
K11	1	21	0.047619048
K12	2	17	0.117647059
K13	1	11	0.090909091
K14	0	12	0
K15	0	9	0
K16	1	15	0.066666667

No. of Proliferating Endothelial Cells (ECs) and Proliferation Index – CAN Nephrectomy

Specimen number	No. proliferating ECs	Total no. proliferating cells	Proliferation Index
UB 011928M/00	3	25	0.12
UB 020768A/95	5	22	0.227272727
UB 018837C/94	4	19	0.210526316
UB 017742C/02	3	20	0.15
UB 003798M/99	4	26	0.153846154
UB 019010F/00	8	26	0.307692308
UB 005718S/02	6	30	0.2
UB 006779/90	1	15	0.066666667
UB 011293B/01	4	29	0.137931034
UB 017246P/00	9	33	0.272727273
UB 023572K/94	3	22	0.136363636
UB 018005S/99	2	18	0.111111111
UB 021907/95	5	23	0.217391304
UB 014924S/00	5	27	0.185185185
UB 001909C/98	3	21	0.142857143
UB 012066A/01	15	51	0.294117647
UB 005247/00	6	24	0.25
UB 023957/01	5	26	0.192307692

Glomerular macrophage infiltration – Normal Kidney

Normal Specimen	No. glomeruli	CD68 count	Index
13394	20	70	3.5
11073	20	47	2.35
11406	20	37	1.85
11837	20	25	1.25
10333	20	78	3.9
9881h	20	34	1.7
9881g	20	43	2.15
9644	20	35	1.75
9493	20	27	1.35
9231	20	57	2.85
8977	20	40	2
7501	20	31	1.55
11940	20	70	3.5
11964	20	29	1.45
11242i	20	62	3.1
11242j	20	54	2.7
12851	20	50	2.5
462	20	38	1.9

Glomerular macrophage infiltration – CAN Nephrectomy

CAN Specimen	No. glomeruli	CD68 count	Index
90/20972	20	40	2
91/20561	n/a	n/a	n/a
93/18110	20	51	2.55
94/18837	10	19	1.9
94/23572	20	31	1.55
95/20768	13	38	2.923076923
96/2921	n/a	n/a	n/a
96/17275	20	40	2
97/3081	20	77	3.85
97/6358	20	43	2.15
98/8353	10	31	3.1
98/12398	20	65	3.25
98/23656	n/a	n/a	n/a
99/3798	20	41	2.05
99/18005	20	46	2.3
99/19767	20	53	2.65
00/5247	20	55	2.75
00/11928	20	35	1.75
00/14924	20	44	2.2
97/18961	14	51	3.642857143
98/1909	20	50	2.5
00/17246	20	73	3.65
00/19010	20	81	4.05
01/11293	20	59	2.95
01/12066	20	62	3.1
01/23079	20	61	3.05
01/23937	20	64	3.2
01/25179	20	77	3.85
002/5718	20	79	3.95

5. Microvessel counts from Renal Allograft Biopsies

MVC comparing Clinical Allograft Biopsy with Normal Kidney - Cortex

Specimen number	Graft biopsy		Specimen	Normal kidney	Specimen	Normal kidney cont.
UB 020921V/91	15		8977	21	K1	25
UB 012868T/91	18		9231	n/a	K2	22
UB 015453R/98	n/a		9493	22	K3	20
UB 004420Q/99	20		9644	23	K4	30
UB 013491F/99	15		10333	23	K5	27
UB 019454/91	17		11073	27	K6	31
UB 019262V/90	20		11406	27	K7	29
UB 003815V/92	n/a		11940	25	K8	20
UB 017016/00	21		11964	22	K9	32
UB 027355Y/98	20		12242i	29	K10	23
UB 019133T/95	18		12242j	20	K11	21
UB 006498/99	20		12851	20	K12	17
UB 026369/98	18		13394	22	K13	20
UB 001832/90	17		462	22	K14	14
UB 114829X/01	23		897	26	K15	25
UB 020812/97	22		899	22	K16	22
UB 008225/01	18		1426	17	K17	20
UB 007465/00	18		2005	21	K18	22
UB 021053/98	19		2226	30	K19	24
UB 09383/94	14		2526	20	K20	n/a
UB 010405/91	18		4152	22	K21	21
UB 016595/95	n/a		4381	22	K22	17
UB 014897/94	n/a		4502	26	K23	23
UB 011967/94	23		5196	24	K24	18
UB 027357D/96	n/a		5192	21	K25	12
UB 014750K/94	21		6552	25	K26	21
UB 011516J/98	n/a		7439	19	K27	20
UB 017819W/95	17		7812	19		
UB 007215Y/01	13		7945	20		
UB 020654F/00	20		8880	21		
UB 002734/95	18		9325	28		
UB 026073/94	n/a		9943	21		
			10083	23		
			10521	21		
			10879	20		

MVC comparing Clinical Allograft Biopsy with Normal Kidney - Medulla

Specimen number	Medullary mvc		Specimen	Normal kidney	Specimen	Normal kidney cont.
UB 020921V/91	12		8977	19	K1	29
UB 012868T/91	14		9231	n/a	K2	30
UB 015453R/98	n/a		9493	20	K3	18
UB 004420Q/99	25		9644	n/a	K4	23
UB 013491F/99	21		10333	25	K5	20
UB 019454/91	n/a		11073	24	K6	17
UB 019262V/90	n/a		11406	19	K7	22
UB 003815V/92	n/a		11940	23	K8	21
UB 017016/00	n/a		11964	n/a	K9	22
UB 027355Y/98	13		12242i	n/a	K10	18
UB 019133T/95	n/a		12242j	n/a	K11	20
UB 006498/99	n/a		12851	n/a	K12	17
UB 026369/98	n/a		13394	25	K13	13
UB 001832/90	n/a		462	22	K14	20
UB 114829X/01	n/a		897	25	K15	27
UB 020812/97	n/a		899	21	K16	25
UB 008225/01	20		1426	n/a	K17	23
UB 007465/00	15		2005	n/a	K18	21
UB 021053/98	14		2226	29	K19	n/a
UB 09383/94	n/a		2526	18	K20	n/a
UB 010405/91	18		4152	24	K21	n/a
UB 016595/95	17		4381	21	K22	n/a
UB 014897/94	16		4502	25	K23	16
UB 011967/94	22		5196	26	K24	16
UB 027357D/96	12		5192	n/a	K25	n/a
UB 014750K/94	n/a		6552	n/a	K26	n/a
UB 011516J/98	18		7439	18	K27	22
UB 017819W/95	13		7812	18		
UB 007215Y/01	n/a		7945	19		
UB 020654F/00	n/a		8880	21		
UB 002734/95	n/a		9325	26		
UB 026073/94	16		9943	17		
			10083	24		
			10521	22		
			10879	17		

Cortical MVC comparing Clinical Biopsies according to Time after Transplantation (months)

Timing after Tx	<12	>12 <36	>36		<12	>12
MVC	17	15	17		17	15
	21	18	14		21	18
	20	20	13		20	20
	18	15	23		18	15
	18	20			18	20
	18	19			18	19
	23	21			23	21
	17				17	17
	20				20	14
	18				18	13
	22				22	23

MVC comparing All CAN Nephrectomy with All Clinical Biopsy – Cortex

Patient initial	Specimen number	Nephrectomy		Patient initial	Specimen number	Biopsy
R S	UB 018110R/93	14		R S	UB 020921V/91	15
H R	UB 011928M/00	14			UB 012868T/91	18
M C	UB 020768A/95	18		H R	UB 015453R/98	n/a
A D	UB 018837C/94	12			UB 004420Q/99	20
J G	UB 017742C/02	13			UB 013491F/99	15
A K	UB 003798M/99	16		M C	UB 019454/91	17
G M	UB 019010F/00	18		A D	UB 019262V/90	20
A M	UB 005718S/02	16			UB 003815V/92	n/a
M S	UB 016885J/02	n/a		J G	UB 017016/00	21
M S	UB 006779/90	17		A K	UB 027355Y/98	20
M B	UB 011293B/01	13		G M	UB 019133T/95	18
B G	UB 017246P/00	18			UB 006498/99	20
A C	UB 026006T/02	22		M S	UB 026369/98	18
G G	UB 023656F/98	13		M S	UB 001832/90	17
F L	UB 017275K/96	17		T L	UB 114829X/01	23
R B	UB 023572K/94	15			UB 020812/97	22
M H	UB 018005S/99	11		M B	UB 008225/01	18
M H	UB 021907/95	16		B G	UB 007465/00	18
A J	UB 020561H/91	14			UB 021053/98	19
A J	UB 018961G/97	12		R B	UB 09383/94	14
D K	UB 014924S/00	14			UB 010405/91	18
N M	UB 020972L/90	13		A D	UB 016595/95	n/a
D M	UB 024483B/00	15			UB 014897/94	n/a
J O	UB 001909C/98	14		M H	UB 011967/94	23
R R	UB 012398L/98	12		A J	UB 027357D/96	n/a
A S	UB 002921C/96	14		J O	UB 014750K/94	21
A D	UB 012066A/01	15		R R	UB 011516J/98	n/a
H W	UB 003081/97	12		A S	UB 017819W/95	17
AJ D	UB 006358/97	18		A D	UB 007215Y/01	13
J D	UB 008353/98	21			UB 020654F/00	20
A C	UB 019767/99	12		H W	UB 002734/95	18
G D	UB 005247/00	16		E M	UB 026073/94	n/a
E M	UB 023079/01	15				
T L	UB 023957/01	15				
W M	UB 025179/01	18				

MVC comparing All CAN Nephrectomy with All Clinical Biopsy – Medulla

Patient initial	Specimen number	Nephrectomy		Patient initial	Specimen number	Biopsy
R S	UB 018110R/93	n/a		R S	UB 020921V/91	12
H R	UB 011928M/00	14			UB 012868T/91	14
M C	UB 020768A/95	14		H R	UB 015453R/98	n/a
A D	UB 018837C/94	n/a			UB 004420Q/99	25
J G	UB 017742C/02	15			UB 013491F/99	21
A K	UB 003798M/99	18		M C	UB 019454/91	n/a
G M	UB 019010F/00	15		A D	UB 019262V/90	n/a
A M	UB 005718S/02	17			UB 003815V/92	n/a
M S	UB 016885J/02	16		J G	UB 017016/00	n/a
M S	UB 006779/90	15		A K	UB 027355Y/98	13
M B	UB 011293B/01	14		G M	UB 019133T/95	n/a
B G	UB 017246P/00	14			UB 006498/99	n/a
A C	UB 026006T/02	23		M S	UB 026369/98	n/a
G G	UB 023656F/98	14		M S	UB 001832/90	n/a
F L	UB 017275K/96	14		T L	UB 114829X/01	n/a
R B	UB 023572K/94	11			UB 020812/97	n/a
M H	UB 018005S/99	22		M B	UB 008225/01	20
M H	UB 021907/95	13		B G	UB 007465/00	15
A J	UB 020561H/91	15			UB 021053/98	14
A J	UB 018961G/97	14		R B	UB 09383/94	n/a
D K	UB 014924S/00	16			UB 010405/91	18
N M	UB 020972L/90	n/a		A D	UB 016595/95	17
D M	UB 024483B/00	17			UB 014897/94	16
J O'	UB 001909C/98	18		M H	UB 011967/94	22
R R	UB 012398L/98	18		A J	UB 027357D/96	12
A S	UB 002921C/96	19		J O	UB 014750K/94	n/a
A D	UB 012066A/01	12		R R	UB 011516J/98	18
H W	UB 003081/97	11		A S	UB 017819W/95	13
AJ D	UB 006358/97	13		A D	UB 007215Y/01	n/a
J D	UB 008353/98	17			UB 020654F/00	n/a
A C	UB 019767/99	14		H W	UB 002734/95	n/a
G D	UB 005247/00	12		E M	UB 026073/94	16
E M	UB 023079/01	16				
T L	UB 023957/01	13				
W M	UB 025179/01	11				

MVC comparing specific CAN Nephrectomy and Clinical Biopsy pairs – Cortex

Patient	XS MVC cortex	Biopsy MVC cortex
1	14	18
2	14	20
3	16	20
4	14	21
5	14	17
6	15	20
7	18	17
8	12	20
9	13	21
10	18	18
11	17	17
12	13	18
13	18	19
14	15	18
15	16	23
16	12	18
17	15	22

MVC comparing specific CAN Nephrectomy and Clinical Biopsy pairs – Medulla

Patient	XS MVC medulla	Biopsy MVC medulla
1	14	25
2	18	20
3	14	12
4	19	13
5	14	20
6	18	19
7	11	18
8	13	22
9	14	15
10	14	18
11	18	18
12	13	16
13	16	16

No. of Proliferating Endothelial Cells (ECs) Comparing CAN Nephrectomy with Clinical Biopsy

Specimen number	No. proliferating ECs - CAN nephrectomy		Specimen number	No. proliferating ECs - Antecedent biopsy
UB 011928M/00	3		UB 004420Q/99	6
UB 020768A/95	5		UB 019454/91	4
UB 018837C/94	4		UB 019262V/90	2
UB 017742C/02	3		UB 017016/00	2
UB 003798M/99	4		UB 027355Y/98	3
UB 019010F/00	8		UB 019133T/95	5
UB 005718S/02	6		UB 006498/99	5
UB 006779/90	1		UB 001832/90	2
UB 011293B/01	4		UB 020812/97	3
UB 017246P/00	9		UB 008225/01	4
UB 023572K/94	3		UB 007465/00	7
UB 018005S/99	2		UB 021053/98	6
UB 021907/95	5		UB 010405/91	3
UB 014924S/00	5		UB 011967/94	4
UB 001909C/98	3		UB 007215Y/01	8
UB 012066A/01	15		UB 020654F/00	5
UB 005247/00	6			
UB 023957/01	5			

Glomerular macrophage infiltration – Early Clinical Biopsy

Specimen number	Earliest Bx glomeruli	Bx count	Bx Index
UB 021053/98	7	9	1.285714
UB 010405/91	13	12	0.923077
UB 011683B/93	1	1	1
UB 020654F/00	15	33	2.2
UB 014897/94	9	16	1.777778
UB 015453R/98	7	5	0.714286
UB 019454/91	4	11	2.75
UB 027355Y/98	8	12	1.5
UB 001832/90	3	8	2.666667
UB 017016/00	5	13	2.6
UB 019133T/95	4	7	1.75
UB 008225/01	5	12	2.4
UB 014750K/94	10	22	2.2
UB 002734/95	11	13	1.181818
UB 020812/97	10	14	1.4

Glomerular macrophage infiltration – Late Biopsy

Specimen number	Later Bx glomeruli	Bx Count	Bx index
UB 021053/98	7	16	2.285714
UB 09383/94	10	18	1.8
UB 007215Y/01	7	13	1.857143
UB 013491F/99	2	2	1
UB 006498/99	3	7	2.333333
UB 114829X/01	10	16	1.6

MVC from Protocol Biopsies – All counts

Tx No.	Bx Date	Bx Day	CAN?	Total glomeruli	Cortical MVC
527	31/07/1985	20	Y	14	22
531	02/09/1985	7	N	12	22
533	03/03/1986	155	Y	2	15
558	17/01/1986	10	Y	10	20
558	29/01/1986	21	Y	7	21
605	28/11/1986	90	N	n/a	n/a
619	20/02/1987	90	N	4	16
721	26/09/1988	210	N	4	15
755	05/10/1988	24	N	3	20
755	16/09/1988	5	N	3	16
755	21/09/1988	10	N	1	14
757	28/09/1988	11	N	6	23
758	18/10/1988	30	N	n/a	n/a
758	23/09/1988	5	N	3	15
758	04/10/1988	16	N	n/a	n/a
759	07/10/1988	16	N	n/a	n/a
759	16/10/1988	25	N	1	5
764	21/10/1988	13	Y	n/a	n/a
764	24/10/1988	16	Y	1	13
766	27/11/1988	32	Y	n/a	n/a
766	02/11/1988	7	Y	7	24
766	09/11/1988	14	Y	4	22
840	22/08/1989	7 years	Y	n/a	n/a
894	10/04/1990	60	Y	3	19
898	19/03/1990	7	N	2	18
898	01/04/1990	20	N	4	17
898	16/04/1990	35	N	n/a	n/a
903	04/05/1990	28	N	3	14
904	04/05/1990	22	N	3	18
905	20/04/1990	6	N	3	20
905	31/05/1990	44	N	4	17
906	01/05/1990	16	Y	n/a	n/a
906	04/05/1990	19	Y	n/a	n/a
911	05/06/1990	27	N	n/a	n/a
915	25/05/1990	5	N	6	25
915	10/06/1990	21	N	3	18
918	04/07/1990	9	N	3	14
921	12/06/1990	6	Y	n/a	n/a
944	10/09/1996	6 years	N	2	18
965	31/01/1991	34	Y	7	25
1067	05/04/1992	4 years	N	n/a	n/a
1092	24/06/1992	7	N	n/a	n/a
1351	22/08/1996	300	GN	3	14
1362	26/02/1996	29	N	1	8
1383	18/06/1996	50	Y	6	24

1391	05/07/1996	28	N	5	16
1395	01/07/1996	14	N	n/a	n/a
1404	30/08/1996	26	N	5	17
1404	29/08/1996	18	N	6	19
1406	28/08/1996	10	N	5	19
1406	11/09/1996	28	N	7	21
1408	20/09/1996	30	N	1	14
1413	20/09/1996	10	N	10	23
1454	29/11/1997	8 month	N	3	15

MVC from protocol biopsies – Stable graft function (<6 month, >3 glomeruli)

Tx No.	Bx Date	Bx Day	CAN?	Total glomeruli	Cortical MVC
531	02/09/1985	7	N	12	22
619	20/02/1987	90	N	4	16
755	05/10/1988	24	N	3	20
755	16/09/1988	5	N	3	16
757	28/09/1988	11	N	6	23
758	23/09/1988	5	N	3	15
898	01/04/1990	20	N	4	17
903	04/05/1990	28	N	3	14
904	04/05/1990	22	N	3	18
905	20/04/1990	6	N	3	20
905	31/05/1990	44	N	4	17
915	25/05/1990	5	N	6	25
915	10/06/1990	21	N	3	18
918	04/07/1990	9	N	3	14
1391	05/07/1996	28	N	5	16
1404	30/08/1996	26	N	5	17
1404	29/08/1996	18	N	6	19
1406	28/08/1996	10	N	5	19
1406	11/09/1996	28	N	7	21
1413	20/09/1996	10	N	10	23

MVC from protocol biopsies – CAN (<6 month, >3 glomeruli)

527	31/07/1985	20	Y	14	22
558	17/01/1986	10	Y	10	20
558	29/01/1986	21	Y	7	21
766	02/11/1988	7	Y	7	24
766	09/11/1988	14	Y	4	22
894	10/04/1990	60	Y	3	19
965	31/01/1991	34	Y	7	25
1383	18/06/1996	50	Y	6	24