MOLECULAR MECHANISMS OF CELL DEATH AND REGENERATION FOLLOWING ISCHAEMIC RENAL INJURY

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TO JENNIFER, JAMES AND JESSICA
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ABBREVIATIONS

ADP      Adenosine diphosphate
AMP      Adenosine monophosphate
ANOVA    Analysis of variance
ARF      Acute renal failure
ATN      Acute tubular necrosis
ATP      Adenosine triphosphate
b-PG     Biotinylated proteoglycan
BUN      Blood urea nitrogen
CTP      Cytosine triphosphate
DAB      Diaminobenzidine
DD-PCR   Differential display polymerase chain reaction
DEPC     Diethylpyrocarbonate
cDNA     Complimentary deoxyribonucleic acid
DNA      Deoxyribonucleic acid
DT       Distal tubules
EDTA     Ethylenediamine tetra acetic acid
EGF      Epidermal growth factor
GFR      Glomerular filtration rate
GTP      Guanine triphosphate
HGF      Hepatocyte growth factor
ICAM-1   Intracellular adhesion molecule-1
IGF-I     Insulin-like growth factor-1
mRNA     Messenger ribonucleic acid
MMLV     Maloney mouse leukaemia virus
MSH      Melanocyte stimulating hormone
MTAL     Medullary thick ascending limb
NAD      Nicotinamide adenine dinucleotide
NO       Nitric oxide
cNOS     Constitutive nitric oxide synthetase
iNOS     Inducible nitric oxide synthetase
NTP      Nucleotidyl triphosphate
PBS      Phosphate buffered saline
PARP     Poly ADP-ribose polymerase
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RT</td>
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<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>TNF</td>
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<td>TNFR</td>
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<td>TTP</td>
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PUBLICATIONS FROM THIS THESIS

Induction of calcyclin after ischemic injury to rat kidney.

Expression of CD27 and ischemia/reperfusion-induced expression of its ligand Siva in rats kidneys.
B. J. Padanilam, **A. J. P. Lewington** and M. R. Hammerman.

Molecular mechanisms of cell death and regeneration in acute ischemic renal injury.
B. J. Padanilam and **A. J. P. Lewington**.
Current Opinion in Nephrology and Hypertension 1999; 8: 15-19.

Expression of CD44 in kidney following acute ischemic injury in rats.

Inhibition of poly(ADP-Ribose) polymerase attenuates ischemic renal injury in rats.
CHAPTER 1

INTRODUCTION

1.1 Normal kidney structure and function

The kidneys perform a variety of essential functions which include the maintenance of body composition, excretion of metabolic end products and foreign substances and the secretion of enzymes and hormones. The kidneys regulate the volume of fluid in the body, its osmolarity, electrolyte content and acidity through the excretion of water and electrolytes. The anatomical structure of the kidney has developed to enable maximal water conservation. The renal medulla, through a countercurrent system of vessels and tubules enables the concentration of urine up to four times the osmolality of the plasma. In health the kidneys correct the perturbations in the composition and volume of body fluids that occur secondary to food intake, metabolism and environmental factors. The kidneys excrete a number of products of metabolism such as urea, and are also involved in the removal of a number of toxins and drugs. Numerous bodily functions depend on the regulatory mechanisms employed by the kidneys. It is important to appreciate the renal anatomy and circulation along with its regulatory functions to better understand the pathophysiology of ischaemic renal injury.

1.1.1 Renal anatomy

The kidneys lie in the retroperitoneal space and weigh about 150 g each. The nephron is the basic functional unit, consisting of a glomerulus and a long tubule lined by a continuous layer of epithelial cells. Both kidneys contain approximately one million nephrons which are segmented into distinct parts, each possessing its own specialised functional characteristics. The glomeruli are located in the outer part of the kidney, called the cortex, whereas the tubules are present in both the cortex and the inner part of the
kidney, the medulla. The medulla is formed into cone-shaped regions called pyramids which extend into the renal pelvis.

The excretory function of the kidney begins with the filtration of plasma across the glomerulus (Figure 1.1). The filtrate then passes into the proximal tubule. The proximal tubule is composed of an initial convoluted segment and a later straight segment, the pars recta or S3 segment, which enters the outer medulla. Approximately two-thirds of the glomerular filtrate is absorbed prior to entering the loop of Henle. The loop of Henle dips down in a hairpin configuration into the medulla before returning to the cortex where it approaches its parent glomerulus. It is here that the specialised tubular cells of the macula densa are located. The tubular fluid then enters the distal convoluted tubule and finally the collecting duct, which courses back through the medulla to empty into the renal pelvis. Along the tubule, most of the glomerular filtrate is reabsorbed with some additional substances secreted. The resultant product is urine which is drained by the ureters into the bladder.

1.1.2 Renal circulation

The kidneys receive their arterial blood supply from a single renal artery (in 80% of cases) branching directly from the aorta. Each kidney is supplied with approximately one-fifth of the cardiac output, which represents the highest tissue-specific blood flow in the body in relation to organ weight. The renal circulation is unusual in that it is divided into two separate capillary beds, known as the glomerular bed and the peritubular capillary bed. These two capillary networks are arranged in series so that all the renal blood flow passes through both. Blood arrives at the glomerulus via the afferent arteriole, where the plasma ultrafiltrate is formed prior to leaving via the efferent arteriole. The efferent arteriole vessels then bifurcate to form the peritubular capillary network. It is at this second
Figure 1.1 Diagram illustrating the components of a nephron and its collecting duct. aa-afferent arteriole; ea-efferent arteriole; gc-glomerular capsule; pct-proximal convoluted tubule; prt-pars recta, S3 segment of proximal tubule; cd-collecting duct; loh-loop of Henle; ts-thin segment, loh; tal-thick ascending limb, loh; dct-distal convoluted tubule; act-arched collecting tubule. (Reproduced from Williams G and Mallik NP. Colour Atlas of Renal Diseases 1994; 2nd ed: 10).
network of capillaries where the tubular filtrate is reabsorbed into the circulation. The peritubular capillary networks found in the outer cortex are related mainly to the proximal tubules. In contrast the efferent arterioles of the inner cortex pass down into the medulla forming a hairpin configuration, the vasa-recta, around the descending S3 segments before rising with the medullary thick ascending limb (MTAL) of Henle to join the venous system. A countercurrent mechanism of exchange exists in the medulla to enable the maximal concentration of urine. Oxygen diffuses from the arterial to the venous vasa-recta which results in the outer medulla being deficient in oxygen. Both the S3 segment and the MTAL are metabolically active with high transport activity which further contributes to the marginal degree of oxygenation in the medulla. The S3 segment in comparison to the MTAL has little glycolytic capacity. Autoregulation maintains a relatively constant renal blood flow to the kidney over a wide range of arterial blood pressures. Loss of the autoregulatory mechanism occurs during episodes of severe hypotension, resulting in a reduction of renal perfusion pressure and consequently glomerular ultrafiltration pressure.

1.1.3 Renal physiology

The main function of the kidneys is the maintenance of a stable body composition. Numerous bodily functions only proceed optimally if the body fluid composition and volume are maintained within an appropriate range. For example enzymatic function is best over a narrow range of pH whilst cell membrane potentials depend on potassium ion concentration. This balance is achieved by the filtration of blood across the glomerulus to form an ultrafiltrate of plasma followed by the secretion and reabsorption of fluid and electrolytes across the tubule epithelial cells lining the renal tubule to produce urine. The movement of fluid and electrolytes is vectorial and occurs across a polarised tubule epithelium. The membrane facing the epithelial tubule lumen is referred to as the apical membrane whereas the lumen facing the blood is referred to as the basolateral membrane.
In the polarised tubule epithelial cells the transport proteins are located either in the apical or the basolateral membrane. The polarity and integrity of the tubule epithelium is maintained in part by the tight junctions. Transport of electrolytes across the cell membrane can be passive or active (energy requiring). Cellular energy is derived from adenosine triphosphate (ATP) generation and hydrolysis. The most important cell transport protein is the Na⁺/K⁺ ATPase pump which extrudes sodium (Na⁺) from the inside of the cell in exchange for potassium (K⁺) from outside the cell. It derives its energy from the hydrolysis of ATP. The Na⁺/K⁺ ATPase pump lies on the basolateral membrane of the renal tubule cell. Activity of this pump is high in the proximal tubule and in the MTAL, where the highest rate of solute transport occurs creating a region of relative hypoxia. It is therefore not surprising that it is these nephron segments that are particularly susceptible to hypoxic injury.

The nephron is divided into several parts each possessing its own functional role. The proximal tubule is responsible for the bulk of the sodium chloride and bicarbonate reabsorption in addition to the almost exclusive reabsorption of glucose, amino acids and small low molecular weight proteins. The loop of Henle enables the kidney to generate concentrated or dilute urine. The distal tubule which joins the collecting duct is composed of a tight epithelium which maintains a steep solute concentration gradient between the lumen and interstitium with minimal movement of water. This segment is also the main site of potassium and hydrogen secretion.

1.2 Acute renal failure

Acute renal failure (ARF) is a life-threatening illness whose mortality has remained high despite advances in modern medicine. Acute renal failure is costly both financially and in terms of morbidity, with a small but significant proportion of patients progressing to end-
stage renal disease and requiring long-term dialysis. It is essential to have a clear understanding of the pathogenesis of ARF, as this will help guide clinicians with the appropriate management. Supportive therapy with dialysis remains the only currently available treatment for ARF.

1.2.1 Definition and incidence of acute renal failure

Acute renal failure (ARF) is characterised by sudden decline in glomerular filtration rate (GFR) resulting in perturbation of extracellular fluid volume, electrolyte and acid-base homeostasis and retention of nitrogenous waste from protein metabolism [Brady H 1996]. Acute renal failure has many different causes although it is often referred to as acute tubular necrosis (ATN). Acute tubular necrosis is strictly a pathological diagnosis and the description should therefore be reserved only for intrinsic ARF, in which there is necrosis of the renal tubule epithelial cells. The majority of cases of ATN are secondary to injury from ischaemia and nephrotoxins, which has led to the term ATN commonly being used to denote ischaemic or nephrotoxic ARF. In most cases of ATN the kidneys are able to recover completely due to the remarkable ability of the tubule epithelial cells to regenerate. The classification and aetiology of the different types of ARF will be outlined later.

Acute renal failure can be oliguric (urine output < 400 mls/day) in 50% of cases, or non-oliguric (urine output > 400 mls/day). The presence of oliguria is associated with more marked histopathological damage. The conversion from an oliguric to a non-oliguric state has been considered beneficial in terms of fluid balance management, a decreased requirement for dialysis and perhaps an improved prognosis [Majumdar S 1996]. The diagnosis of ARF is made on the basis of the clinical presentation and on a rising blood urea and serum creatinine, which are unfortunately, relatively insensitive indices of GFR. In acute renal failure large changes in GFR are initially only manifested as small changes
in serum creatinine. As the GFR decreases there is a concomitant increase in the tubular secretion of creatinine which results in a delay before the serum creatinine truly reflects the reduction in GFR [Moran S 1985].

The true incidence of ARF is hard to estimate as there is no centralised registry of patients with ARF. However, the most comprehensive studies in developed countries indicate that the overall incidence of ARF is around 200 per million population per year [Liano F 1999], of whom 25% require dialysis [Doherty C 1998]. The incidence of ARF increases markedly with age which reflects a decrease in renal functional reserve and exposure to more complex medical and surgical conditions. It is therefore not surprising that the population receiving dialysis is becoming increasingly older. Acute renal failure has been estimated to be present in 1% of patients admitted to hospital [Kaufman J 1991] and occurs in approximately 5% of patients during hospitalisation [Shusterman N 1987]. Its frequency rises in patients on the intensive care unit and can complicate up to 30% of admissions [Hou S 1983]. Acute renal failure remains a major cause of in-hospital morbidity and mortality.

1.2.2 Classification and aetiology of acute renal failure

Acute renal failure is classically divided into three categories (Table 1.1): pre-renal ARF (55%) where there is decreased renal perfusion without cellular injury; intrinsic ARF (40%) with involvement of the renal parenchymal tissue; post-renal ARF (< 5%) associated with acute obstruction of the urinary tract. Pre-renal ARF and intrinsic ARF due to ischaemia and nephrotoxins are responsible for most episodes of ARF. Sustained prerenal ARF as is the most common cause of ischaemia induced ATN [Cameron J 1986]. Acute renal failure occurring in hospitalised patients is usually multifactorial [Davidman M 1991], with combinations including patients with hypotension in the setting of sepsis
and the use of angiotensin converting enzyme inhibitors in patients with renal artery stenosis.
<table>
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<tr>
<th>Pre-renal</th>
<th>Intrinsic</th>
<th>Post-Renal</th>
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<td>- Intravascular volume depletion &lt;br&gt;  o vomiting &lt;br&gt;  o diarrhoea &lt;br&gt;  o poor fluid intake &lt;br&gt;  o diuretics &lt;br&gt;  o burns &lt;br&gt; - Decreased cardiac output &lt;br&gt;  o heart failure &lt;br&gt; - Systemic vasodilatation &lt;br&gt;  o sepsis shock &lt;br&gt; - Renal vasoconstriction</td>
<td>- Intrarenal vascular disease &lt;br&gt; - Glomerulonephritis &lt;br&gt; - Ischaemia &lt;br&gt;  o prolonged prerenal failure &lt;br&gt; - Toxins &lt;br&gt;  o aminoglycosides &lt;br&gt;  o radiocontrast agents &lt;br&gt;  o haem pigments &lt;br&gt;  o chemotherapeutic agents &lt;br&gt;  o myeloma light chains &lt;br&gt; - Interstitial nephritis &lt;br&gt;  o drugs &lt;br&gt;  o autoimmune disease &lt;br&gt;  o infiltrative disease &lt;br&gt; - Intrarenal obstruction &lt;br&gt;  o uric acid &lt;br&gt;  o acyclovir &lt;br&gt; - Renal vessel obstruction</td>
<td>- Ureteric obstruction &lt;br&gt;  o bilateral renal calculi &lt;br&gt;  o bladder carcinoma &lt;br&gt;  o retroperitoneal fibrosis &lt;br&gt; - Bladder neck obstruction &lt;br&gt;  o prostate &lt;br&gt;  o cervix &lt;br&gt; - Urethral obstruction &lt;br&gt;  o stricture</td>
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**Table 1.1** Causes of acute renal failure.
**Pre-renal acute renal failure**

Prerenal acute renal failure is the most common cause of ARF being responsible for up to 55% of patients presenting with this condition. It can complicate any disease that is associated with 'true' hypovolaemia (e.g. haemorrhage, vomiting, diarrhoea, burns) or decreased 'effective' circulating volume (e.g. low cardiac output, liver failure, sepsis). The kidneys are able to preserve perfusion pressure during episodes of mild hypoperfusion through several compensatory mechanisms [Badr K 1988]. However during states of severe hypotension these compensatory renal responses are overwhelmed resulting in a reduction in renal perfusion pressure and loss of glomerular filtration rate. By definition the integrity of the renal parenchymal tissue is maintained in pre-renal acute renal failure, and the GFR recovers rapidly upon restoration of haemodynamic stability. Failure to restore systemic blood pressure resulting in a prolonged or severe episode of renal hypoperfusion is the most common cause of ischaemic ATN.

**Intrinsic acute renal failure**

Intrinsic acute renal failure is responsible for about 40% of patients presenting with ARF. It can be categorised according to the primary site of injury: renal vessels, glomeruli, tubules or interstitium. In 90% of cases of it is the proximal tubules that are damaged through ischaemia or toxic insults.

Pre-renal failure progresses to ischaemic acute renal failure, ATN, when the renal perfusion is reduced to such an extent that tubule epithelial cell death occurs. Most cases of ischaemic acute renal failure are potentially reversible if the underlying causes are corrected, and haemodynamic stability restored. After ischaemia, toxins account for the next most common cause of intrinsic acute renal failure. The list of potential nephrotoxins is extensive but includes aminoglycosides, radiocontrast agents, chemotherapeutic drugs,
myeloma light-chain proteins and haem pigments. In the hospital setting ischaemia and toxins will often combine to cause acute renal failure in patients whose illness is complicated with conditions such as sepsis or haematological cancers.

Allergic reactions to a drug are the most common cause of acute renal failure due to an interstitial process (interstitial nephritis). Other causes include severe infections (e.g. legionnaire's disease) and infiltrative diseases such as sarcoid, lymphoma or leukaemia. Acute renal failure secondary to acute interstitial nephritis is often reversible after the withdrawal of the offending medication or treatment of the underlying disease. Acute renal failure due to vascular disease is infrequent but has been described in association with atheroemboli, thrombosis, dissecting aortic aneurysm or vasculitis. A vasculitic process may also affect the renal microvasculature and often involves the glomeruli in the form of a rapidly progressive glomerulonephritis. Non-inflammatory diseases of the microvasculature can cause ARF and include accelerated hypertension and thrombotic microangiopathies such as haemolytic-uraemic syndrome and thrombotic thrombocytopenic purpura.

*Post-renal acute renal failure*

Acute renal failure occurs when there is obstruction to the urinary outflow system to both kidneys or to the urinary outflow tract of a single functional kidney. Urinary tract obstruction accounts for less than 5% of cases of ARF. The most common causes include prostatic enlargement, cancer of the cervix or retroperitoneal disease. During the early stages of obstruction there is increased intraluminal pressure resulting in the gradual distension of the renal pelvis and calyces and a fall in GFR. Arterial vasoconstriction further contributes to a decline in glomerular filtration. Prompt diagnosis and treatment of
obstruction is crucial since the potential for recovery of renal function is inversely related to the duration of the obstruction [Shapiro S 1976].

1.3 Pathology of ischaemic acute renal failure

It is only through understanding the mechanisms that underlie the development of ATN at the cellular and subcellular level that new therapeutic strategies can be designed. The current concepts regarding the mechanisms of cellular injury and reduction in GFR post-ischaemic renal injury are derived predominantly from studies in experimental animals and in-vitro systems. These models mimic the pattern of injury seen in human ARF, although it is well accepted that no experimental model fully replicates the process of renal injury in humans. However, the value of in-vitro models should not be underestimated as they do represent a first approximation of clinical injury and have led to some central morphological observations, as well as demonstrating the presence of multiple factors that potentially contribute to the pathophysiology of ischaemic renal injury. In common with many disease processes it is likely that there are a number of different mechanistic pathways that act in concert to result in ARF in any individual patient.

1.3.1 Morphology of ischaemic acute renal failure

Human renal biopsy and autopsy studies have shown that in common with animal models of ischaemic renal injury the straight segment of the proximal tubule (S3 segment or the pars recta) is a site of significant injury following ischaemia [Bohle A 1976, Dunnill MS 1974, Oliver J 1951, Solez K 1979]. Other morphological features that are shared between human biopsy samples and animal models include diffuse effacement and thinning of the proximal tubular brush border and the presence of intraluminal casts, which are composed of protein and cellular debris. Areas of cellular regeneration are often present together with areas of cellular necrosis in the same biopsy specimen.
A well-recognised paradox of ischaemic ARF is the severity of renal impairment that can occur in the face of relatively subtle histological changes [Racusen LC 1992]. The use of experimental models has provided further insights into this phenomenon and has suggested that sublethal injury to tubule epithelial cells plays an important role in ischaemic renal injury [Lieberthal W 1996]. The most extensively studied experimental model of ischaemic ARF involves the induction of renal ischaemia through renal artery clamping. In this model it is the S3 segment of the proximal tubule that is most severely damaged with lesser damage identified in the distal tubules [Lieberthal W 1998]. The injury is typically diffuse with its severity related to the duration of ischaemia, with a period of 60 minutes being associated with severe renal failure [Finn WF 1979]. The characteristic features include the early loss of the brush border followed by extensive necrosis involving the entire proximal tubule and intraluminal casts formation. Blebs of brush border that are shed into the lumen continue to swell as they travel down the tubules resulting in the obstruction of proximal and distal tubule segments. These histological changes are similar to those described following ischaemic ARF in humans, and provide a rationale for using this model.

1.4 Pathophysiology of ischaemic acute renal failure

Conceptually it became clear over 20 years ago that cell injury not only occurs during the period of ischaemia but also occurs during the reperfusion phase following ischaemia [Paller MS 1994]. The realisation that events that had previously believed to have already been sustained during the ischaemic period may not yet have taken place was initially anticipated to have important therapeutic potential. It was hoped that the cell injury occurring during this period may not only be treatable but could actually be preventable. Despite this promise there has been little overall clinical impact. It has become clear that although a substantial proportion of cell injury occurs at reperfusion, another substantial
part of the injury is sustained during the ischaemic period itself, and therefore cannot be affected by intervention at the time of reperfusion. The relative proportions that the two components of ischaemia and reperfusion contribute to the total post-ischaemic injury vary among different organs, under different conditions, and with varying times of ischaemia. In experimental models it is difficult to separate injury produced by ischaemia per se from that which occurs during reperfusion. It can be argued that the separation is somewhat arbitrary and artificial because recovery from ischaemia is impossible unless reperfusion occurs. However it is useful to consider briefly the predominant effects that each of these pathways exert separately before developing a composite description of the cellular and haemodynamic events that occur following ischaemic injury.

Complete renal artery occlusion results in ischaemic injury secondary to a combination of diminished oxygen delivery in addition to impaired delivery of other nutrients to cells and reduced washout of metabolic waste products. The most prominent effect of ischaemia is the cessation of oxidative phosphorylation with the ensuing depletion of adenosine triphosphate (ATP), and impaired cellular function [Bonventre JV 1993, Weinberg JM 1991].

Reperfusion of the ischaemic organ results in the sudden restoration of oxygenation and the generation of oxygen free radicals [Greene EL 1991]. Oxygen free radicals are detrimental because of their high reactivity with a wide variety of biomolecules, including lipid containing cell membranes. Lipid peroxidation follows the exposure to oxygen free radicals, with disruption of structural integrity of the lipid membrane and increased membrane permeability [Southern PA 1988]. A more detailed description of these events follows later in this chapter.
Pivotal to the induction of ischaemic ARF is the development of both abnormalities in intrarenal haemodynamics and renal tubule epithelial cell dysfunction [Thadhani MD 1996]. It is these two major mechanisms that are responsible for the profound reduction in GFR post-ischaemic renal injury. Intrarenal vasoconstriction results from an imbalance between the local and systemic vasoconstrictive and vasodilative factors. Epithelial cell injury leads to the back leak of glomerular filtrate through the abnormally permeable, denuded tubular basement membrane and thereby compromises GFR. In addition the development of intraluminal casts results in tubular obstruction and a further reduction in GFR.

1.4.1 Haemodynamic alterations in ischaemic acute renal failure

As previously discussed ischaemic injury particularly affects the S3 segment of the proximal tubule and to a lesser extent the MTAL which both lie in the region of the outer medulla. The outer medulla of the normally perfused kidney is constantly in a hypoxic state supplied with a relatively low oxygen tension ($pO_2$ of 1.3-2.7kPa) compared to the oxygen tension in the cortex ($pO_2$ of 6.7kPa). The low oxygen tensions are secondary to the active solute transport performed by the proximal tubule and the MTAL. The medulla receives only 20% of total renal blood flow which is important in maintaining adequate medullary hypertonicity for urinary concentration. Medullary hypertonicity is generated through the countercurrent exchange mechanism and shunting of oxygen from arterial to venous limbs of the vasa recta, which further contributes to the hypoxic state [Levy MN 1961]. Thus, it is not surprising that a reduction in blood flow may result in ischaemic injury to the outer medulla [Brezis M]. It is important to recognise the capacity of the S3 segment and the MTAL to generate energy (ATP) by glycolysis in the context of susceptibility to ischaemic injury. The proximal tubules have little capacity for glycolysis, in contrast to the superior glycolytic capacity of the MTAL which provides it with a
greater capacity to generate ATP than the S3 segment during periods of renal ischaemia [Bagnasco S 1985].

A reduction in total renal blood flow to 40% to 50% of normal has been consistently reported in both experimental models and in the maintenance phase of ATN in humans [Brady H 1996]. Studies using Doppler probes demonstrate that after 60 minutes of ischaemia blood flow in the superficial cortex falls to 60% of pre-ischaemic levels and to 16% of pre-ischaemic levels in the outer medulla, but increased to 125% of control values in the inner medulla [Hellberg O 1990]. Despite the restoration of renal blood flow post-ischaemia it has been demonstrated that the outer medulla remains profoundly hypoxic. Haemodynamic abnormalities play an important role in ischaemic injury by causing persistent regional disturbances in renal blood flow and a reduction in the oxygen supply to the outer medulla of the kidney following ischaemia, the no-reflow phenomenon [Mason J 1984]. This contributes to renal dysfunction through the maintenance of a hypoxic state in the region most severely effected by the ischaemic insult. Minimally invasive intra-vital microscopy of blood flow through peritubular capillaries has provided evidence of the existence of the no-reflow phenomenon following ischaemic injury to rats [Yamamoto T 2002]. Transplantation of endothelial cells or surrogate cells expressing endothelial nitric oxide synthase into the renal microvasculature resulted in protection of renal function following ischaemic injury [Brodsky SV 2002]. This supports the theory that endothelial dysfunction is the primary cause of the no-reflow phenomenon after ischaemic renal injury. It has been proposed that there are at least two independent factors at play, namely intrarenal vasoconstriction and the physical congestion of the medullary vasculature that occur post-ischaemic injury.
Intrarenal vasoconstriction

The mechanisms that are important for maintaining autoregulation of renal blood flow remain incompletely defined, but there is evidence that endothelin is an important mediator of vasoconstriction [Kon V 1989] as well as tubule epithelial cell injury [Chan L 1994]. It is the most potent endogenous vasoconstrictor described to date, possessing prolonged activity and exhibiting a preferential action on the renal vasculature [Kohan D 1993]. Endothelin receptor blockers improve renal function and ameliorate the morphologic severity of tubular injury associated with ischaemic ARF [Gellai M 1994, Huang C 2002]. It has been suggested that ischaemic injury induces the upregulation of endogenous endothelin which then is capable of perpetuating its own production resulting in the long-lasting vasoconstriction that occurs post-ischaemic ARF. Conversely endothelial injury decreases the release of nitric oxide (NO) produced by the constitutive nitric oxide synthase (cNOS) [Lieberthal W 1989]. The constitutive production of NO plays an important role in regulating vascular tone by maintaining basal systemic and renal arterial vasodilatation [Baylis C 1993]. In addition, NO acts to down-regulate the production and activity of endothelin in vitro. More recently it has become clear that the role of NO in ischaemic ARF is complex, as NO produced by ischaemic tubular epithelial cells mediates cellular injury [Yu L 1994]. In this case the NO is generated by inducible nitric oxide synthase (iNOS) an enzyme distinct from cNOS. There is therefore compartmentalisation of the nitric oxide system whereby reduced endothelium-derived NO production causes vasoconstriction and worsens ischaemia, whilst increased NO production by tubule epithelial cells exacerbates the injurious effects of ischaemia. Thus endothelial injury in ischaemic ARF leads to an imbalance in the vasoconstrictive and vasodilative actions of endothelin and NO respectively and helps to explain the intrarenal vasoconstriction that occurs.
In addition to the imbalance between the vasoconstrictor and vasodilator mechanisms, ischaemic injury to vascular smooth muscle cells of the kidney has been demonstrated to result in disorganisation of the actin cytoskeleton component F-actin [Kwon O 2002]. It has been proposed that this disruption of the actin cytoskeleton may contribute to the loss of autoregulation of renal blood flow and the aberrant vascular reactivity following ischaemia.

**Medullary congestion**

Ischaemic cellular damage results in swelling of tubule epithelial cells and endothelial cells leading to vascular congestion, which together with external compression from interstitial oedema further exacerbates the reduction in renal blood flow experienced within the outer medulla. Congestion and physical obstruction of the capillaries of the outer medulla occurs secondary to the trapping of red cells platelets and leucocytes [Hellberg O 1991]. The leucocyte-endothelial adhesion results in events similar to those that occur with a classic inflammatory reaction the contribution of which will be described further later. The importance of red cell trapping has been demonstrated in a study in which a reduction in the systemic haematocrit greatly reduces medullary congestion and ameliorates ischaemic injury [Hellberg O 1990].

1.4.2 *Mechanisms of cell injury in ischaemic acute renal failure*

Tubule epithelial cell injury in ischaemic ARF plays a major role in the reduction of GFR. Two important pathogenic mechanisms have been described which are the development of tubule leakiness and tubule obstruction. Ischaemic injury results in the detachment of tubule epithelial cells and debris which together form obstructing casts in the tubules. Micropuncture studies in experimental models have demonstrated elevated proximal and distal intratubular pressures and a marked reduction in single nephron glomerular filtration
rate. The injured cells that become detached leave a denuded basement membrane that allows the leakage of tubular fluid back into the peritubular capillaries. Tubule leakiness has been demonstrated through the injection of molecular markers into the tubules of ischaemic kidneys, which later appear in the urine of the contralateral uninjured kidney [Donohoe JF 1978]. Oliguria and prolonged renal ischaemia are associated with a greater degree of tubule leakiness. Evidence from renal biopsy and autopsy studies supports the importance of these mechanisms in human ARF. Therefore damage to the tubule epithelial cell represents a central event underlying the pathophysiology of ischaemic ARF. A number of different pathogenic mechanisms have been demonstrated to be involved in this injurious process which will be discussed further.

**ATP depletion**

Renal ischaemia is accompanied by an abrupt fall in adenosine triphosphate (ATP) levels, with a greater reduction in the cortical levels of ATP in comparison to medullary levels [Gerlach E 1971]. After 1 minute of renal ischaemia the whole kidney ATP content decreases by 70%, and after 10 minutes the ATP content has decreased to less than 10% [Hems DA 1977]. It is the proximal tubule segments of the nephron that mainly lie in the cortical region and which depend predominantly on the mitochondria for ATP production. Such observations provide support for the hypothesis that the differences in susceptibility of the cortex and medulla of the kidney to ischaemic injury are related not only to the differences in regional reperfusion that occur but also to the differences in regional ATP requirements that exist. This helps to explain why it is the proximal tubule that is generally more susceptible to ischaemic renal injury than other nephron segments. In particular it is the S3 segments of the proximal tubule that lie in the relatively hypoxic environment of the outer medulla, which sustain most severe injury.
A wide variety of cellular processes depend on the hydrolysis of the high-energy phosphate of ATP. These processes include protein synthesis, lipogenesis and membrane transport. A reduction in cellular ATP as occurs in ischaemia results in dissipation of ion gradients dependent on the ATPase transporter systems and an accumulation of sodium and calcium. Acidosis will develop as the cell switches to glycolytic metabolism. Breakdown of ATP results from ischaemia and leads to the formation of adenosine diphosphate (ADP) and adenosine monophosphate (AMP), to which epithelial cell membranes are relatively permeable. Rapid restoration of ATP levels is therefore possible if the length of ischaemic injury is short. However prolonged renal ischaemia will result in the metabolism of AMP to the nucleosides adenosine, inosine and hypoxanthine which can all leak out of cells depleting the purine substrate pool. Furthermore these purines can constrict intrarenal arterioles and through their further metabolism result in the formation of reactive oxygen species [Bonventre JV 1993]. Prolonged ischaemic injury also results in irreversible loss of mitochondrial function. Therefore central to the ability of the cell to survive ischaemia is the rate of cell ATP recovery which is dependent upon the duration of the ischaemic injury [Weinberg JM 1991].

The importance of the role played by ATP depletion post-ischaemic renal injury has been confirmed by a number of experimental studies. A reduction in cellular injury through the preservation of ATP levels has been demonstrated during ischaemia performed in hypothermic conditions [Lieberthal W 1988]. Proof of the importance of the adenine nucleotide pool in recovery of cellular ATP levels and reduction in cellular injury after ischaemic injury has been shown through the provision of adenosine, inosine and exogenous adenine nucleotides [Siegel NJ 1980].
**Calcium homeostasis**

To maintain the steep gradient that exists between intracellular Ca\(^{2+}\) and extracellular Ca\(^{2+}\), intracellular free calcium Ca\(^{2+}\) concentration is tightly regulated by active transporter mechanisms. This gradient is maintained by both the Ca\(^{2+}\)-ATPases (present in both the plasma membrane and endoplasmic reticulum) and the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger. Following renal ischaemia and ATP depletion there is failure of a number of transmembrane ion transport mechanisms which results in uncontrolled calcium influx [Krippen A 1994]. These include impaired extrusion of Ca\(^{2+}\) from the cell and sequestration of Ca\(^{2+}\) in the endoplasmic reticulum in addition to the failure of the Na\(^+\)/K\(^+\)-ATPase which potentiates intracellular movement of Ca\(^{2+}\) via the Na\(^+\)/Ca\(^{2+}\) exchanger. Once the buffering capacity of the endoplasmic reticulum is exceeded Ca\(^{2+}\) uptake by the mitochondria occurs. This results in mitochondrial swelling and injury with uncoupling of oxidative phosphorylation and a further reduction in ATP levels. Rises in intracellular Ca\(^{2+}\) levels contributes to tubule epithelial cell toxicity by disrupting cytoskeletal microfilaments, activating proteases and phospholipases and facilitating the generation of reactive oxygen species.

Whether increased intracellular Ca\(^{2+}\) is the actual cause of tubule epithelial cell injury rather than merely being a result of the ischaemic injury remains controversial. Central to this argument is whether cytosolic or mitochondrial Ca\(^{2+}\) increases before lethal or sublethal cell injury occurs. Currently this question remains unresolved, however the evidence available does suggest that increased intracellular Ca\(^{2+}\) is an important event in the evolution of ischaemic cellular injury [McCoy CE 1988, Schwertschlag U 1986, Snowdowne KW 1985].
Reactive oxygen species

Reperfusion following ischaemic renal injury is associated with the rapid formation of reactive oxygen species (ROS). In the kidney these oxidants are produced by a number of sources which include cyclo-oxygenases, mitochondrial electron transport, mixed-function oxidases of the endoplasmic reticulum, the xanthine oxidase system and neutrophils. Reactive oxygen species exert a number of deleterious effects on cells that include lipid peroxidation, oxidation of cell proteins and cellular DNA damage [Ichikawa I 1994]. A number of studies have demonstrated a role for ROS in ischaemic renal injury firstly through the detection of cellular markers of oxidant injury and secondly through the amelioration of renal ischaemic injury using both inhibitors of ROS production as well as ROS scavengers [Lieberthal W 1990]. However other observations have raised concerns regarding the role of oxidant injury during ischaemic/reperfusion injury and at present there is not sufficient evidence to support the use of ROS inhibitors or scavengers in patients with acute renal failure [Johnson KJ 1993].

Phospholipases

Phospholipase A2 is a family of enzymes that hydrolyse phospholipids to free fatty acids and lysophospholipids and has been proposed to play an important role in ischaemic cellular injury [Bonventre JV 1993]. Phospholipase A2 contributes to ischaemic tissue injury in the kidney through a variety of different actions. Phospholipid degradation results in alterations in plasma membrane and mitochondrial membrane permeability, as well as the production of arachidonic acid whose metabolites are vasoactive and chemotactic for neutrophils. In addition the conversion of arachidonic acid to eicosanoids generates reactive oxygen species. Protein kinase C activation may enhance phospholipase A2 activity by phosphorylating one isoform of this enzyme, thereby acting as a positive feedback loop for enhanced phospholipase A2 activity [Nemenoff RA 1993]. Further
advances in the role of phospholipases in the pathogenesis of ischaemic renal injury have been held back due to a lack of specific inhibitors.

**Neutrophils and reperfusion injury**

It has been demonstrated that neutrophils play a pivotal role in the reperfusion phase following ischaemic injury in a number of different organ systems. Following ischaemia and during the period of reperfusion neutrophils adhere to the vascular endothelium prior to extravasation into the surrounding tissue [Springer TA 1994]. The resulting neutrophil activation results in the release of reactive oxygen species, proteases, elastases, myeloperoxidase and other enzymes that can lead to tissue damage. There have been a number of studies post-ischaemic renal injury some of which support a pathogenic role of neutrophils [Hellberg O 1990, Klausner JM 1989], and others that do not [Paller MS 1989]. The most interesting work concerns the use of monoclonal antibodies against intracellular adhesion molecule-1 (ICAM-1) to attenuate neutrophil recruitment and functional renal impairment in rats subjected to renal ischaemia [Kelly KJ 1994]. In this study anti-ICAM-1 monoclonal antibody was protective when administered at the time of induction of ischaemia or even up to 120 minutes following the injury. Histologically there was a marked reduction in ischaemia-induced increases in tissue myeloperoxidase, a marker of neutrophil infiltration. Subsequently it has been demonstrated that mice with a deficiency of ICAM-1 are protected against acute ischaemic renal failure [Kelly KJ 1996]. ICAM-1 is normally prominent in the endothelium of the vasa recta in normal mice. After induction of ischaemic injury renal leucocyte infiltration was markedly less in the mutant mice than control mice. The mutant mice were protected from acute renal ischaemic injury as judged by serum creatinine, renal histology and animal survival.
Complement activation

Studies have shown that complement activation contributes to the pathogenesis of renal injury following ischaemia [Thurman JM 2003, Zhou W 2000]. Complement activation is an early event in the course of ischaemia/reperfusion injury with the generation of complement effector molecules influencing the function of other factors such as free radicals, neutrophils and the products of activated endothelium [Kilgore KS 1994]. Complement activation in the kidney following ischaemic injury appears to occur exclusively via the alternative pathway, as demonstrated by the protection afforded to mice deficient of complement factor B, an essential component of the alternative pathway, when rendered ischaemic [Thurman JM 2003]. Mice deficient in the complement factor 4 are also protected from ischaemic injury lending further support to the importance of the alternative pathway [Zhou W 2000]. Furthermore it has been identified that the primary effect of complement activation is on the tubule epithelial cell rather than on the vascular endothelial cell, which differs from the site injury seen in other organ systems [Zhou W 2000].

1.4.3 Cytoskeletal injury and tubular obstruction

The actin cytoskeleton plays an important role in mediating a myriad of processes necessary for cellular structure and function. Actin cytoskeletal-surface membrane interactions mediate a diverse range of cellular events which include maintenance of cell polarity, endocytosis, exocytosis, cell division, cellular migration, cell adhesion, signal transduction and ion channel activity. ATP is required for the regulation and maintenance of the cell cytoskeleton [Molitoris BA 1991]. Ischaemic renal injury results in ATP depletion and profound alterations in actin ultrastructure leading to loss of epithelial cell polarity, disruption of the brush border and impaired function of the cellular tight junctions. There is loss of cell-cell and cell-substrate adhesion secondary to a redistribution
of transmembrane proteins such as the β1 integrins, and resultant detachment of viable cells from their matrix. Restoration of ATP levels before lethal cell injury occurs allows the recovery of the normal actin cytoskeletal architecture and cell function [Kroshian VM 1994]. It would therefore appear that disruption of the actin cytoskeleton is a marker of sublethal cell injury which is a potentially reversible preterminal event.

Renal tubular obstruction is a recognised cause of renal dysfunction in ischaemic acute renal failure and is contributory to delayed recovery of renal function [Arendshorst WJ 1976]. Human and experimental studies have demonstrated the presence of viable renal tubular epithelial cells in the urine during periods of acute renal failure. It has been postulated that desquamated sublethally injured cells attach to each other and to cells remaining in-situ via integrin receptors resulting in tubular obstruction. Support for this theory has been provided by a study demonstrating that short peptides bearing the amino acid motif of integrin receptors was able to inhibit cast formation and functional renal impairment in experimental ischaemic acute renal failure [Goligorsky MS 1993].

1.5 Sublethal cell injury

In the kidney tubule epithelial cells respond to ischaemic injury in a number of different ways (Table 1.2). The fate of a proximal tubule epithelial cell during ischaemic injury is dependent upon the severity and the duration of the ischaemic insult (Figure 1.2) [Finn WF 1979]. Some cells will escape injury completely, while others are sublethally injured and are capable of complete recovery either directly or through an undifferentiated cell intermediate if the insult is removed in time [Lieberthal W 1996]. Lethally injured cells may die in a process of necrosis or apoptosis, depending upon the severity of the injury to which the cell is exposed [Lieberthal W 1988]. The predominant form of cell death after ischaemic renal injury is necrosis. However morphologic studies of renal tissue after
Figure 1.2 The fate of a proximal tubule cell following ischaemic injury. The severity and duration of the ischaemic injury determines the fate of a proximal tubule cell. Sublethal cell injury is followed by either direct recovery or recovery through an undifferentiated cell intermediate. Lethal cell injury can occur rapidly by necrosis or in a more delayed fashion by apoptosis. (Reproduced from Sutton TA and Molitoris BA). Mechanisms Of Cellular Injury In Ischemic Acute Renal Failure. Seminars in Nephrology 1998; 5: 490-497).
ischaemic injury in humans have in some cases revealed that areas of frank necrosis are focal and relatively sparse despite profound reductions in renal function. This has led to the proposal that a significant number of tubule epithelial cells are sublethally injured. Such a population of sublethally injured cells while appearing intact morphologically contribute to the tubular leakiness and obstruction that occurs in ischaemic acute renal failure.

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<td>• Altered gene expression</td>
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<td>• Cellular dedifferentiation</td>
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<td>• Recovery of cell function</td>
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<th>Lethal injury</th>
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<tr>
<td>• Necrosis</td>
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<td>• Apoptosis</td>
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Table 1.2 Potential responses of tubule epithelial cells to ischaemic injury

Sublethal injury is associated with disruption of the actin cytoskeleton which is responsible for a number of the functional disturbances identified in renal tubule epithelial cells post-ischaemia [Kroshian VM 1994]. In health the actin cytoskeleton is important in maintaining normal brush border structure, cell polarity, tight junction integrity and normal cellular adhesion. ATP depletion post-ischaemic renal injury results in a loss of cell polarity due to failure of the "fence" function of the tight junction, and also to a "back leak" of glomerular filtrate due to failure of the "gate" function of the tight junction [Molitoris BA 1997]. At this stage the injury sustained is reversible if the ATP levels are restored promptly. In addition sublethally injured tubule epithelial cells undergo a loss of cell matrix adhesion. This has been proposed to be due to a redistribution of cell matrix

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adhesion proteins, in particular the β1 integrin, with a further consequence being the aberrant binding of tubule epithelial cells to one another resulting in tubular cast formation and obstruction [Goligorsky MS 1993]. Recovery of renal function and structure is dependent upon a series of events which includes the recovery of sublethally injured cells and repopulation of denuded areas of basement membrane. During this process sublethally injured cells undergo altered gene expression, cellular dedifferentiation, proliferation and ultimately recovery of cell function.

1.6 Necrotic cell death

Acute tubular necrosis (ATN) describes the mode of cell death to which the most severely injured cells succumb post-ischaemia. Necrosis is accompanied by massive tissue damage and rapid collapse of the internal homeostasis of the cell. The earliest morphological changes that occur with ischaemic tubule epithelial cell injury include the loss of the apical brush border and blebbing of apical membranes. With more advanced ischaemic injury there is loss of plasma membrane integrity, vacuoles form within the cell, mitochondria swell and nuclei undergo pyknosis. Cells detach from the basement membrane, leaving gaps, and the remaining cells flatten out along with the basement membrane [Racusen LC 1997]. Cellular debris and intratubular protein form casts and obstruct the tubules causing increased tubular pressure proximal to the obstruction. Cell necrosis is secondary to overwhelming and prolonged ATP depletion which results in irreversible injury to the plasma membrane lipid bilayer and subcellular organelles [Bonventre JV 1993]. The loss of cell membrane integrity has led to the use of "vital dyes" such as trypan blue to assess cell viability. Cells possessing intact membranes are impermeant to these dyes, whereas the dyes can diffuse freely into necrotic cells. Disruption of cell membrane structure and transporter activity leads to cell swelling and leakage of proteolytic enzymes into the extracellular space. Histologically a strong local inflammatory response can be easily
identified in tissue sections. The molecular events that result in necrosis are poorly defined but occur in a chaotic and uncoordinated manner. DNA degradation occurs secondary to digestion by proteases and endonucleases resulting in a smear pattern when viewed on an agarose gel, since the proteases destroy the histones and expose the entire length of DNA to the nucleases [Wyllie AH 1993]. Some of the potential mechanisms that underlie the process of necrosis are listed below (Table 1.3)

<table>
<thead>
<tr>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe ATP depletion</td>
</tr>
<tr>
<td>Membrane ion transport pump dysfunction</td>
</tr>
<tr>
<td>Cell swelling</td>
</tr>
<tr>
<td>Activation of phospholipases</td>
</tr>
<tr>
<td>Activation of proteases</td>
</tr>
<tr>
<td>Increased intracellular free calcium</td>
</tr>
<tr>
<td>Plasma and subcellular membrane injury</td>
</tr>
</tbody>
</table>

**Table 1.3** Mechanisms of cell necrosis

### 1.7 Apoptotic cell death

In direct contrast to necrosis, apoptosis is a carefully orchestrated form of cell death that requires energy [Hockenbery D 1995]. Apoptosis was first described in 1972 by Kerr et al who defined apoptosis using morphological criteria and clearly demonstrated that necrosis and apoptosis can be differentiated by distinct phenotypic characteristics (Table 1.4). The term apoptosis is Greek in origin and describes the dropping off or falling off of petals from flowers, or leaves from trees. Morphological examination is undoubtedly the most reliable method for distinguishing between apoptotic and necrotic cell death [Hockenbery
D 1995, Majno G 1995]. The characteristics of cell death secondary to apoptosis are unique and remain constant in many different cell types. Cells dying from apoptosis become smaller secondary to a reduction in cytosolic volume as well as condensation of nuclear chromatin [Kerr JFR 1972, Savill J 1995, Ueda N 1994]. The cell membrane retains its structural integrity, and subcellular organelles remain morphologically normal. Cells undergoing apoptosis remain impermeable to vital dyes and also retain their cytosolic contents. Consequently unlike necrosis there is little evidence of surrounding tissue injury or inflammation.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Membrane permeability</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>Membrane budding</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Chromatin</td>
<td>Condensed</td>
<td>Normal</td>
</tr>
<tr>
<td>DNA appearance</td>
<td>Ladder</td>
<td>Smear pattern</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Normal</td>
<td>Swollen</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Cell fate</td>
<td>Phagocytosis</td>
<td>Lysis</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Absent</td>
<td>Present</td>
</tr>
</tbody>
</table>

**Table 1.4** Morphological differences between apoptotic and necrotic cell death

Following a reduction in cell volume a process of plasma membrane "budding" occurs which is a consequence of cell membrane disconnection from the underlying cytoskeleton [Bright JJ 1994, Martin SJ 1994]. Multiple "apoptotic bodies" are produced which are membrane bound vesicles containing condensed chromatin and cytosolic organelles. Transmission electron microscopy demonstrates intact plasma membrane and subcellular organelles as well as nuclear condensation. These apoptotic bodies are then rapidly phagocytosed by resident macrophages and surrounding epithelial cells and fibroblasts [Cohen JJ 1993, Corcoran GB 1994]. It has been estimated that the entire process of apoptosis takes approximately 2 hours from start to finish with the rapid removal of apoptotic cells through phagocytosis making apoptotic cells potentially difficult to detect.
on histological sections [Wylie AH 1980, 1994]. It should therefore be realised that apoptosis can be responsible for extensive cell loss despite its inconspicuous nature on histological section. The visualisation of even small percentages of stained apoptotic cells in-situ yields biologically significant data, often unobtainable by examination of histochemically stained tissue or by DNA ladder assays. Apoptosis not only serves for the rapid clearance of dying cells but also protects the surrounding tissues from injury and inflammation that occurs from necrotic cell death.

A number of biochemical events have now been identified in addition to the morphological changes described above. Endonuclease activation occurs that results in the production of DNA fragments in integer multiples of 200 base pairs. DNA is most vulnerable to the effects of endonucleases at internucleosomal sites, where DNA is not protected by histones. Agarose gel electrophoresis of the DNA demonstrates a characteristic "ladder" pattern [Bortner CD 1995]. DNA fragmentation associated with apoptosis has also been demonstrated using end labelling with fluorescently labelled deoxyuridine of the free double-stranded ends of DNA using the enzyme terminal deoxynucleotidyl transferase (TdT) [Gavrieli Y 1992]. This technique using TdT-mediated d UTP nick end labelling (TUNEL) method has provided a useful way to identify and quantify cells undergoing apoptotic cell death in tissue sections. The labelling target is the multitude of new 3'-OH DNA ends generated by DNA fragmentation. These are typically localised in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei, which have relatively insignificant numbers of DNA 3'-OH ends do not stain. Cells exhibiting necrotic morphologies can in some instances contain sustainable concentrations of DNA ends, but they appear more diffuse than apoptoses.
1.7.1 The commitment and execution phases of apoptosis

Apoptosis has been divided into two phases, the commitment phase (the time when an individual cell decides to undergo apoptosis) followed by the execution phase (when activation of affecting mechanisms occur) [Earnshaw WC 1995, Steller H 1995]. The commitment phase is stochastic in nature such that following an apoptotic stimulus cells will become committed to apoptosis after a variable and completely random time interval. Cells will therefore die in an asynchronous manner over a variable period of time following a single apoptotic trigger [Earnshaw WC 1995, Gerschenson LE 1992]. It has been proposed that a therapeutic window of opportunity may exist during the commitment phase up to initiation of the execution phase. The execution phase is typically brief and refers to the period during which the cell undergoes the morphological changes of apoptosis and ultimately phagocytosis [Majno G, Steller H 1995]. Evidence so far would indicate that new gene transcription is not required and the proteins necessary for the execution of apoptosis probably reside in a latent state within the cytoplasm [Bertrand R 1994, Earnshaw WC 1995, Enari M 1995, Jacobson MD 1994, Savill J 1995]. It is likely that many features of the cell signalling process leading to apoptosis are shared with those associated with the necrotic form of cell death. The form of cell death and pathway followed is dependent upon the nature and severity of the injury, with the same insult leading to either apoptosis when present in a mild form, or necrosis when present in a more severe form.

Caspases are a family of cell death proteases that play a key role in the execution of apoptosis [Martin SJ 1995]. The term caspase signifies two distinct properties of these enzymes in which "c" refers to the cysteine proteases and "aspase" denotes their specificity to cleave after aspartic acid. So far 14 members of the caspase family have been identified in mammalian cells [Wiegele G 1998]. These enzymes are produced as inactive pro-
enzymes that are activated by cleavage at critical aspartate residues. During the execution phase there is activation of the caspase enzymes in successive and expanding hierarchies. This proteolytic cascade ultimately leads to the activation of proteases that target multiple proteins within the cells. The proteolytic cleavage of the cytoplasmic and nuclear substrates leads to DNA fragmentation and cell death by apoptosis [Martin S 1995]. At least 40 different protein substrates for caspases have been recognised which include DNA repair enzymes such as poly (ADP-ribose) polymerase (PARP), DNA fragmentation factor responsible for internucleosomal DNA cleavage [Gu Y 1995, Tewari M 1995], nuclear structural proteins (lamin) [Martin SJ 1995], cytoskeletal proteins and caspases themselves. The recent description of caspase inhibitors that are capable of inhibiting apoptosis induced by number of stimuli has provided confirmatory evidence that caspase activation is an important proximal event in the apoptotic pathway [Jacobson M 1996].

1.7.2 Genes involved in apoptosis

The products of several gene families have been implicated in the control of apoptosis. Of particular interest is the Bcl-2 family of proteins that is important in regulating the execution phase of apoptosis [Hockenberry DM 1995]. The Bcl-2 protein (Bcl-XL) protects cells against apoptosis induced by a variety of stimuli. Other counterregulatory members of the Bcl-2 family (Bax, Bcl-Xs) have been identified, that bind with one another to form heterodimers. It appears that the balance between cell death and survival following an apoptotic stimulus is determined by the ratio of concentrations of apoptosis-promoting and apoptosis-suppressing Bcl-2 family proteins. During the recovery phase of acute renal failure remodelling of injured renal tubules takes place through a process of renal cell proliferation and differentiation. Evidence exists that apoptosis plays an important role during this repair phase and in particular the Bcl-2 family of proteins [Basile D 1997].
1.7.3 Evidence supporting a role for apoptosis in ischaemic renal injury

There is increasing evidence that apoptosis plays a contributory role in the pathophysiology of acute ischaemic renal injury following ischaemia [Lieberthal W 1996, 1998, Padanilam B 1998, Schumer M 1992, Shimizu A 1993]. Morphologic studies have demonstrated the presence of apoptotic bodies following ischaemic renal injury. In one of the studies the relative degree of cell death from apoptosis was dependent upon the period of ischaemia, with shorter periods of ischaemia demonstrating a greater proportion of cell death from apoptosis in comparison to necrosis. Following more prolonged periods of ischaemia there was increasing evidence of cell death from necrosis but substantial numbers of apoptotic bodies were still present [Schumer M 1992]. A further study not only demonstrated apoptosis occurring 12-48 hours post-ischaemic renal injury in rats but also detected apoptosis occurring during the recovery phase [Shimizu A 1993]. Further work in our laboratory has confirmed the presence of apoptosis by immunohistochemistry using the TUNEL technique occurring 12-24 hours post-ischaemic renal injury and also during the regeneration of the renal tubule epithelium [Padanilam B 1998]. Apoptosis is therefore not only an important cause of cell death immediately following ischaemic injury but also contributes to the recovery and remodelling of the renal tubule by providing a balance for excessive renal cell proliferation. In-vitro studies utilising severe depletion of cellular ATP levels induced by chemical anoxia have also demonstrated cell death in proximal tubular cells secondary to apoptosis [Lieberthal W 1998].

1.7.4 Triggers of apoptosis

The role of apoptosis in ischaemic acute renal injury is less well-defined than in other disease states. A number of different well-established triggers of apoptosis are recognised which all lead to morphologically identical features of apoptosis (Table 1.5). These triggers will be discussed and a potential role in ischaemic renal cell death examined.
Programmed cell death is dependent upon a genetically directed clock that selects a given time for death of certain cells [Eastman A 1993, Gerschenson LE 1992, Majno G 1995]. It is an important mechanism for eliminating unwanted cells during embryogenesis. It plays no role in renal disease once embryogenesis is complete.

Cell surface receptor ligand binding can induce apoptosis. Members of the tumour necrosis factor (TNF) superfamily are the best characterised cell surface receptors capable of mediating cell death by apoptosis. Its members include the Fas (also called CD95 or Apo 1) [Nagata S 1995] and TNFR1 [Baker SJ 1998] receptors that are bound by the Fas ligand (FasL) and TNF-α respectively. As a result of receptor ligation there is a clustering of the receptors' death domains which result in a death inducing signalling complex via the activation of the caspase cascade. Evidence exists for the expression of Fas [Boonstra JG 1997] and TNF-α [Jevnikar AM 1991] on renal tubule epithelial cells. Addition of TNF-α to renal tubule epithelial cells has been shown to induce apoptotic cell death [Taguchi T 1998], whilst there has been increased TNF-α demonstrated in regenerative tubule epithelial cells following ischaemic renal injury [Goes N 1996]. Evidence also exists for a role for Fas-dependent cell signalling in apoptosis post-ischaemia with increased renal tubule epithelial cell expression of Fas and FasL [Nogae S 1998].
Growth factor deficiency induces apoptosis in renal tubule epithelial cells through an imprecisely understood pathway [Lieberthal W 1996]. However there is alteration in the expression of pro-apoptotic and anti-apoptotic genes along with the ligation of cell surface receptors resulting in the activation of the apoptotic pathway. The expression of a number of growth factors which include EGF, IGF-1 and HGF have all been shown to be altered post-ischaemic renal injury and their administration capable of accelerating renal recovery [Hammerman MR 1994]. The beneficial role of growth factor administration may not lie only in accelerating the regenerative process but also through a reduction in cell injury and cell death from apoptosis. Support for this theory has been provided by work demonstrating that HGF inhibits apoptosis when administered following ischaemic renal injury in rats [Vijayan A 2001].
Impaired cell adhesion results in cell death from apoptosis [Frisch SM 1994]. It is believed that survival signals are transmitted to the cells from the environment via integrins or other adhesion molecules that mediate cell-matrix attachment. Cell-cell adhesion has also been shown to act as a survival signal for epithelial cells [Bates RCA 1994].

Cytotoxic stimuli of a wide variety are capable of causing cell injury and cell death from apoptosis or necrosis. Apoptosis is a common response in cells exposed to modest ischaemia or toxins at concentrations below those required to induce metabolic collapse and necrosis. Cytotoxic stimuli include reactive oxygen metabolites [Takeda M 1999] and ceramide [Ueda N 1992] both of which are generated following ischaemic renal injury [Zager RA 1997].

1.8 Regeneration after ischaemic renal injury

The kidney possesses a remarkable propensity for recovery of cellular function and histological morphology following ischaemic acute renal failure, with most patients regaining sufficient renal function for a normal life [Bonventre JV 2003]. Ischaemic injury of the kidney causes sublethal and lethal injury of tubule epithelial cells. Histologic examination demonstrates the detachment of individual or groups of cells from the basement membrane into the tubule lumen exposing gaps in the epithelium [Racusen LC 1997]. Analysis using urine cytology indicates that up to 30% to 50% of the exfoliated renal tubule epithelial cells are considered to be viable by trypan blue exclusion studies. The majority of the cells are of proximal tubule origin, and the long-term viability of these exfoliated tubule epithelial cells has been confirmed by the successful establishment of primary cell cultures [Graber M 1991]. The recovery of renal function following ischaemic injury depends on the recovery of sublethally injured cells, removal of necrotic cells and intratubular casts, cell migration and regeneration of renal cells to restore the normal
continuity and function of the nephron [Toback FG 1992]. Removal of lethally injured cells is achieved by macrophages which infiltrate damaged sites following the ischaemic injury prior to the accelerated phase of proliferative regeneration [Bonventre JV 1993]. It is well-established that wound macrophages have the capacity to produce growth factors and cytokines and these may play a role in modulating the inflammatory response. Surviving cells at the edges of the wound are capable of migrating to cover denuded areas of tubule basement membrane, while cell proliferation is required to provide a sufficient number of migrating cells to re-epitheliasate larger defects [Toback FG 1992]. For cell migration to occur a cell must first detach its connections between cytoskeletal components, integrins, and extracellular matrix proteins such as fibronectin, collagens and laminin which anchor the cell to the tubule basement membrane [Goligorsky MS 1993]. Cells that migrate undergo transcriptional, biochemical, structural, and physiological changes to enable them to participate in wound healing. The cascade of molecular events involved in this process has not yet been defined. It is possible from the evidence accumulated so far that remaining intact or sublethally injured tubule epithelial cells secrete growth factors that act in an autocrine or paracrine manner to facilitate migration, recovery and/or proliferation of cells at the wound edge [McNeill PL 1989]. Once re-epitheliasation has been achieved cell migration ceases and the formation of a mature functional epithelium occurs with the development of cell-matrix adhesions and cell-cell junctions.

Under physiologic conditions of approximately one cell per human nephron is sloughed into the urine each day [Prescott LF 1966]. Therefore under normal circumstances there is some proliferation of renal tubule epithelial cells required to maintain the structural integrity of the kidney. That cell proliferation occurs during acute renal failure despite the retention of toxins, acidosis, electrolyte imbalance and reduced nutrient intake is a
testament to the extraordinary capacity of renal cells to regenerate. During this process, normally quiescent renal cells dedifferentiate, increase their nucleic acid synthesis and undergo mitosis [Bonventre JV 2003]. Such a response is typical following all forms of acute renal injury including ischaemia. The factors that induce dormant renal tubule epithelial cells to re-enter the cell cycle and undergo mitosis are still being elucidated. The renal DNA synthetic response to injury likely entails the co-ordinated interplay of a number of cellular genes responding to stimuli transduced within the cell. It is likely that a set of intracellular signalling events occurs ultimately leading to the transcription of genes whose encoded proteins mediate the response to ischaemic injury. Activation of signal transduction pathways will result in responses such as proliferation, growth arrest, hypertrophy, differentiation or cell death. Many of these intracellular signalling events converge on a set of cellular kinase cascades which are collectively called the mitogen-activated protein (MAP) kinase pathways [Bonventre JV 1998, Di Mari JF 1999]. These kinase pathways and others are critically important for the transcriptional activation of a number of genes. Prominent among these activated genes are the so-called immediate early genes whose expression is enhanced almost immediately following stimulation by many growth factors [Bravo R 1990]. Some of these immediate early genes encode DNA-binding transcription factors that likely initiate a genetic cascade ending in cell division. This gene induction is transient and independent of new protein synthesis. Two such immediate early genes that are expressed within the first few hours after ischaemia are c-fos and Egr-1 [Safirstein R 1994]. The c-fos and Egr-1 proteins contain DNA binding motifs and regulate the transcription of other genes. Paradoxically the expression of these proteins is induced in the medullary thick ascending limbs, where there is less extensive damage and regeneration during the post-ischaemic period. In fact there are a number of genes whose expression demonstrates both positive and negative regulation after ischaemic injury in the MTAL [Megyesi J 1995, Padanilam BJ 1996, Safirstein R 1994]. These genes
code for a number of protein products which include cytokines and growth factors, leading to the suggestion that these molecules might be secreted by the MTAL to act on the injured S3 segment of the proximal tubule which lies in close proximity. It is unclear whether the MTAL response is to a self generated signal or to one arising from outside the cell membrane. Following ischaemic renal injury the majority of cell proliferation and regeneration occurs in the S3 segment of the proximal tubule. Proliferating cell nuclear antigen (PCNA) is a DNA polymerase-associated protein that can be used as a marker for the G1-S transition in the cell cycle and hence mitogenesis. Immunohistochemistry has demonstrated PCNA positive nuclei primarily in the S3 segment occurring maximally at 24-48 hours following ischaemic renal injury in the rat [Witzgall R 1994]. The same population of surviving tubule epithelial cells expressing PCNA in the S3 segment also stained positively for vimentin indicating cell dedifferentiation. It has been proposed that this data supports the hypothesis that the mature renal S3 segment epithelial cell can be a progenitor cell.

It is clear that the reaction of tubule epithelial cells to ischaemic injury is heterogeneous. Some cells will die while others will survive and commit to repair of the injured epithelium. The determinants of these responses will likely depend on signal transduction pathways and molecular responses that are specific and interactive between different segments of the kidney. Several changes occur in gene expression which suggest that the kidney may recapitulate a developmental programme and differentiate, at least partially, in order to commit to DNA synthesis [Toback FG 1992].

The pattern of gene expression following ischaemic renal injury bears a striking resemblance to that exhibited by growth factor stimulated cells in culture [Safirstein R 1994]. This has led to an interest in the role growth factors may play in the regenerative
process, and their potential therapeutic benefit. Increasing evidence has suggested that growth factors may play an important role in nephrogenic repair following acute renal failure [Humes HD 1995, Schena FP 1998]. There are a number of growth factors that are synthesised within the kidney and can stimulate renal tubule epithelial cell mitogenesis. Initially, the surviving tubule epithelial cells express chemotactic factors that attract inflammatory cells [Frank J 1993, Safirstein R 1991]. The recruited inflammatory cells and injured resident tubule epithelial cells synthesise and release growth factors that act in a paracrine and autocrine manner to facilitate the proliferative repair process. The expression and therapeutic action of three of these growth factors, in particular, have been studied in acute renal failure which include epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF).

1.8.1 Epidermal growth factor

Epidermal growth factor (EGF) is a single chain peptide of 53 amino acids which is a potent renal epithelial cell mitogen. The EGF gene is normally expressed in the distal nephron and loop of Henle, whilst its receptor is found on the basolateral membrane of the proximal tubule and collecting duct. Following an ischaemic insult the renal expression of prepro EGF falls as does the total renal content of EGF [Schaudies RP 1993], however the number of renal EGF receptors increases [Safirstein R 1994]. Experimental work has demonstrated that when EGF is administered to animals following ischaemia renal tubule cell regeneration and repair is enhanced and the recovery of renal function is accelerated [Humes HD 1989]. In contrast administration of EGF to normal controls does not provoke renal DNA synthesis indicating that the increase seen during acute renal failure may be dependent upon the increase in EGF receptor number.
1.8.2 Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is a polypeptide comprised of 70 amino acids with a high degree of homology to proinsulin. In rat kidney immunoreactive IGF-1 is localised primarily in the medullary collecting duct [Bortz JD 1988]. Within 6 hours of ischaemic renal injury in-situ hybridisation and immunohistochemistry have localised IGF-1 mRNA transcripts and the IGF-1 peptide to the regenerative zones of renal injury [Matejka GL 1992]. A similar pattern of expression for the IGF-1 receptor was appreciated. Regenerating cells showed an upregulation of the IGF-1 receptor density by 50-70% at 48-72 hours after the ischaemic insult, with a return to baseline levels of expression at 7 days post-ischaemia. The administration of IGF-1 in animal models of ischaemic renal failure accelerates the rate of renal recovery [Miller SB 1992]. The exact mechanism of this protection is unclear however histological evaluation showed fewer pathological changes when compared with untreated controls, and intrarenal haemodynamics were improved with an increase in glomerular filtration rate and renal blood flow. Of particular interest is that the protective effect is observed even when the IGF-1 is administered 24 hours after the ischaemic renal insult in rats. Such success in animal experiments has led to human trials of the therapeutic potential of IGF-1. In patients undergoing surgery involving the suprarenal aorta and surgery involving the renal arteries administration of IGF-I reduced the incidence of postoperative renal dysfunction [Franklin SC 1997]. More recently a multicentre clinical trial of recombinant human IGF-1 in patients with acute renal failure failed to accelerate the recovery of renal function [Hirschberg R 1999].

1.8.3 Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a heterodimeric glycoprotein, which is a very potent mitogen for renal tubule epithelial cells as well as mature parenchymal hepatocytes [Igawa T 1991]. In addition to its cell growth regulatory activity, HGF enhances cell motility
In the kidney HGF mRNA has been localised using in-situ hybridisation to the mesangial cells and interstitial macrophages. Within 6 hours of ischaemic renal injury there is increased expression of HGF within the kidney. The HGF receptor, the c-met proto-oncogene, also demonstrates increased expression following ischaemia within the glomerular and proximal tubule epithelium [Igawa T 1993]. Expression of c-MET is most marked in the most severely damaged nephron segments in the outer medulla, where DNA synthesis is also most prominent [Rabkin R 2001]. Exogenous HGF has been shown to stimulate recovery after acute renal injury in rats [Miller SB 1994]. This effect could be achieved by a variety of mechanisms which include the mitogenic and motility enhancing properties of HGF along with its ability to act as a survival factor. Indeed, HGF has now been demonstrated to inhibit apoptosis in this model [Vijayan A 2001].

1.8.4 Role of extra-renal cells in regeneration

The discovery of pluripotent bone marrow-derived stem cells has led to a re-examination of the cellular source and processes involved in the recovery from organ injury [Alison MR 2000, Krause DS 2001]. The kidney has no recognisable stem cell zone, however it has been demonstrated that adult mouse neural stem cells injected in to an early embryo contribute to the developing kidney [Clarke D 2000]. Data has started to accumulate indicating that bone marrow cells contribute to both normal turnover and regeneration after damage of renal epithelial cells [Gupta S 2002, Lin F 2003, Poulsom R 2001]. It has been demonstrated using in situ-hybridisation that Y-chromosomes are present in the cells of kidneys of female mice that had received a male bone marrow transplant [Poulsom R 2001]. Further work by the same group in humans has demonstrated the presence of Y-chromosome-positive cortical tubular epithelial cells in female kidneys transplanted into male recipients.
Haemopoietic stem cells have now been shown to contribute to the regeneration of renal tubules after ischaemic injury to mice [Lin F 2003]. In this study haemopoietic stem cells that express beta-galactosidase were transplanted immediately following unilateral renal ischaemic injury. Four weeks following this procedure beta-galactosidase-positive cells were detected in renal tubules of the recipients. Human studies have provided further support for the role of extra renal cells in regeneration following acute renal failure [Gupta S 2002]. The examination of tubule epithelial cells in male patients with resolving acute tubular necrosis, who had previously received a kidney transplant from a female donor demonstrated the presence of Y-chromosomes-positive cells. No Y-chromosome-positive tubule epithelial cells were identified in similar sex mismatched transplants who did not develop ATN, suggesting that recipient derived cells do not routinely repopulate the transplanted kidney.

1.9 Complications and outcomes of acute renal failure

Acute renal failure impairs the renal excretion of electrolytes and water. As a result ARF is frequently complicated by intravascular volume overload, hyperkalaemia, hyponatraemia, hyperphosphataemia, hypocalcaemia, hypermagnesaemia and metabolic acidosis. Retention of nitrogenous waste products occurs which may result in the development of the uraemic syndrome. The severity of these complications reflects the severity of the renal injury. Acute renal failure has a significant impact on extra-renal organs, with only minor perturbations in renal function conferring excess mortality that is independent of other factors [Levy EM 1996]. This particular study suggested that a more complicated relationship exists between renal failure, comorbidity and death than is currently appreciated. Renal failure increased the likelihood of death from pre-existing non renal complications, and also increased the likelihood that important non renal conditions would occur. Patients with ARF are at an increased risk of acquired sepsis,
bleeding, coma and respiratory failure. Renal dysfunction results in impaired host defences, bone marrow suppression, coagulopathy, cardiovascular dysfunction and central nervous system depression. Systemic increases in the inflammatory mediators tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL -1) after renal ischaemia in the mouse have been reported [Kelly KJ 2003]. Further work has demonstrated increased levels of immunoreactive TNF-α and IL-1 and intercellular adhesion molecule-1 (ICAM-1) mRNA in the heart after renal ischaemia in the rat. This was accompanied by increases in myeloperoxidase activity, an index of tissue leucocyte infiltration, in the heart as well as the liver and lung. Evidence of apoptosis of cardiac cells was also found, which was limited by blocking the action of TNF-α [Kelly KJ 2003]. Leucocyte accumulation has been observed in the liver and spleen of mice following ischaemic injury [Miyazawa S 2002]. The accumulated intermediate T-cells in the liver possessed cytotoxic activity and were associated with the liver dysfunction. It has therefore been proposed that a systemic cellular immune response affects multiple organs during ischaemic acute renal failure, which may play an important role in the development of multiorgan failure.

Acute renal failure remains the most serious and expensive renal disorder in medical practice and appears to be increasing in frequency. Despite developments in treatment the overall mortality rate from ARF has not substantially altered in the past 40 years [Turney JH 1992]. It has been proposed that this apparent lack in improvement in outcome is related to the fact that the patients treated today are older, have significant comorbid conditions and frequently have multiple organ failure. Outcome information is unfortunately not always uniformly measured and varies among the reported series available in the literature. Despite this the available data indicates that the outcome in ARF remains very disappointing with a mortality rate ranging from 40 to 50% in general series to around 70 to 80% in the intensive care setting. In general most patients (57 to
72%) who survive ARF secondary to ATN achieve a complete recovery. Some patients will have residual renal impairment, however they retain enough function to remain dialysis independent for the remainder of their lives. Acute renal failure is irreversible in approximately 5% of patients, usually as a result of complete cortical necrosis, and requires a long-term renal replacement therapy. In contrast about 30% of survivors of severely ill patients who required dialysis in the intensive care setting will need long-term dialysis. Prevention of acute renal failure is therefore important and its early detection and total management essential to reducing mortality. A worse outcome has been observed when there has been a delay in requesting a nephrological opinion [Mehta R 1995].

2.0 Management of acute renal failure

The initial care of patients with acute renal failure is focused on reversing the underlying cause and correcting fluid and electrolyte imbalances. Considerable care should be made to avoid further insults to the kidney and supportive measures should be provided until recovery has occurred. More specific therapy is dependent upon the underlying cause of the acute renal failure.

Haemodialysis remains the major supportive intervention in severe acute renal failure, which fails to resolve with conservative management. Retrospective studies have shown that providing renal replacement therapy with dialysis is certainly better than having no dialysis [Kleinknecht D 1972, Fischer RP 1996]. However dialysis itself is a procedure not without a risk. Associated with its initiation are the risks of bleeding and later infection from the site of vascular access. There is evidence indicating that dialysis may delay recovery of renal function from ATN through the activation of inflammatory cascades by the blood dialyser interface [Arnaout MA 1985], or through the occurrence of inter-dialytic hypotension. Experimental models have shown that there is impaired renal autoregulation
in response to hypotension during acute renal failure and therefore the kidney is at increased risk of further ischaemic injury during dialysis. Until recently there has been no consensus as to the optimum time to commence dialysis or the frequency with which it should be performed. However, there have been a few publications that have shed some light on these issues. A prospective study investigated the effect of daily intermittent haemodialysis, as compared with conventional (alternate-day) intermittent haemodialysis on survival among patients with acute renal failure in medical and surgical intensive care units [Schiffl H 2002]. This study demonstrated that those patients receiving intensive haemodialysis had a reduced mortality without any increase in haemodynamically induced morbidity. In agreement with this study was the publication of a prospective randomised trial which looked at the impact of different ultrafiltration doses in continuous renal replacement therapy on survival [Ronco C 2000]. Patients with acute renal failure in the intensive care unit setting were enrolled. The mortality among these critically ill patients was high, but an increased in the rate of ultrafiltration improved survival significantly. Such results suggest that adequate renal replacement therapy is probably one of a number of factors that are important in affecting the outcome of acute renal failure in critically ill patients.

2.1 Summary and aims of research

The mortality from acute renal failure has changed very little over the last four decades remaining at about 50%. Ischaemic acute renal failure remains the leading cause of intrinsic acute renal failure in adults [Thadhani R 1996]. Its course is variable ranging from a transient disease associated with full recovery of renal function to a disease of longer duration requiring dialysis and intensive care management. Previous studies have suggested that acute renal failure is merely an epiphenomenon that reflects the severity of other medical problems. More recently it has been demonstrated that the development of
even mild ARF itself increases mortality [Levy EM 1996]. There also exists a correlation between the duration of the kidney dysfunction and mortality from acute renal failure. Such findings should prompt us to resist from regarding ARF as just a treatable complication of a serious illness. Despite advances in modern medicine the only therapy available for acute renal failure remains supportive with the mainstay of treatment being dialysis. However, evidence exists that dialysis itself may cause renal injury that prolongs renal failure. This may be caused by episodes of hypotension or activation of the inflammatory cascade by the blood-dialyser interface. Animal studies have shown that kidneys with acute renal failure have impaired autoregulation and are therefore at increased risk to recurrent injury from further episodes of hypotension [Conger JM 1994, 1995]. A better understanding of the pathogenesis of acute renal failure would clearly be beneficial and may allow the development of new therapeutic interventions that would shorten the course of the disease and improve survival.

The pathophysiology of ARF is complex and incompletely delineated. There exist both vascular and cellular mechanisms responsible for the decrease in GFR and loss of integrity of the nephrons. Intrarenal vasoconstriction continues despite restoration of normal perfusion to the kidney following ischaemia. This is secondary to an imbalance between the vasoconstrictive and vasodilative factors, which may be mediated by systemic or local vasoactive agent activity on the small vessels of the kidney. At the cellular level important biochemical abnormalities include ATP depletion, increased cytosolic calcium concentration, disruption of the actin cytoskeleton and activation of phospholipase and complement [Bonventre JV 1993]. It is crucial to realise that after an episode of ischaemia, reperfusion itself contributes substantially to the injury that occurs. Of particular importance is free-radical-mediated reperfusion injury.
Anatomically it is the straight segment of the proximal tubule (S3 segment) that lies in the outer medulla which suffers the most cellular damage after an ischaemic injury. This region of the kidney is only marginally oxygenated under normal circumstances and is most vulnerable to an ischaemic insult [Brezis M 1995]. It has been demonstrated that the oxygen concentration here falls to 16% of its baseline value during ischaemia. After the initial injury there is increased medullary congestion of the vasa recta which further perpetuates the ischaemia and delays adequate reperfusion of this region. There is sloughing of viable and nonviable cells into the tubular lumen in the formation of casts and luminal obstruction and contributing to the reduction in the GFR. A spectrum of cellular injury occurs post-ischaemic injury, which is dependent on the severity of the insult. This ranges from cellular dysfunction and nonlethal cell injury to cell death from apoptosis and necrosis.

Despite severe damage the kidney retains the propensity to restore its structure and function. The kidney undergoes a regenerative phase during which time there is induction of various genes, dedifferentiation, increased mitotic activity, spreading of viable cells and a contribution from extra-renal cells to re-epithelialise the damaged tubules [Toback FG 1992, Lin F 2003]. Recovery of the kidney post ischaemic injury duplicates certain aspects of renal development. Expression of proteins only normally expressed in the early stages of nephron development occurs. An understanding of the mechanisms responsible for this pattern of expression may lead to therapies designed to potentiate the regenerative response and lessen the duration of ARF. Experimental models of ischaemic acute renal failure have yielded substantial important information on the pathophysiology of renal injury and repair. The role of growth factors in the recovery from acute renal failure has previously been investigated following the demonstration of a change in their gene expression post-ischaemic injury. Epidermal growth factor (EGF), hepatocyte growth factor (HGF) and
insulin-like growth factor-1 (IGF-1) all accelerate the recovery of normal renal function and regeneration of the damaged proximal tubular epithelium, and improve the mortality in post-ischaemic rat tubular injury. Unfortunately despite their demonstrated benefit in the rat model they have not proved as successful in clinical trials. The search therefore continues for new therapeutic strategies to ameliorate the course of acute renal failure.

A number of genetic programmes are activated post-ischaemic injury which mediate a variety of pathways including those leading to cell death and those affecting cell regeneration and repair. The aim of the proposed study is to identify genes whose expression is enhanced by the induction of ischaemic injury, thereby enabling a better understanding of the molecular mechanisms of cell death and regeneration in acute ischaemic renal injury.

Gene expression post-ischaemic injury will be studied in male Sprague-Dawley rats. A variety of techniques including differential display PCR (DD-PCR) [Liang P 1995] and differential acute renal failure library screening will be used to study gene expression. The temporal and spatial expression of gene and gene products will be characterised using in-situ hybridisation and immunohistochemistry to gain insight into their potential role. Further in-vivo studies may be necessary to determine their biological activities.
CHAPTER 2

METHODS

2.1 Model

Acute renal failure due to ischaemic or toxic injury is traditionally referred to as acute tubular necrosis (ATN). Acute tubular necrosis has an unpredictable onset and does not lend itself to human studies. Experimental models have provided us with most of the information available on the functional and cellular events that lead to ATN.

Ischaemic ATN in humans most often occurs in the context of volume depletion, sepsis and shock. A suitable model of sepsis associated with ARF in rats does not exist whilst severe and prolonged hypotension does not induce renal injury in rats [Zager RA 1987]. Many investigators have therefore used rat models of reversible ischaemic tubule damage induced by complete occlusion of the renal artery for various time periods. This model possesses some important pathological similarities to human ATN including injury to the proximal brush border, with a predilection for the proximal straight segments (S3 segments) [Venkatachalam M 1978, Torhorst J 1982] and the presence of tubular cast formation [Bonventre JV 1993, Finn WF 1979, Glaumann B 1977, Kreisberg JJ 1988]. There are also functional similarities with a reduction in GFR and the potential for complete recovery of renal function.

Morphological characteristics of ATN in humans that are less evident in animal models include injury to the distal tubule and in particular the medullary thick ascending limb (MTAL), in the outer medulla [Jones DB 1982, Oliver J 1953, Olsen TS 1990]. Differences also occur between human ATN and animal models of ischaemic renal injury with regard to the degree of tubule cell loss. In animal models there is extensive cellular
necrosis involving the entire proximal tubule structure. In contrast, the major abnormality of ATN described in humans is patchy and focal loss of individual or clusters of cells with resultant denuded basement membrane [Bohle A 1976, Solez K 1979]. However this feature may be difficult to quantitate, because adjacent cells spread to cover these denuded areas, resulting in a flattened and paucicellular appearance to the tubules in cross-section. This reactive response results in rapid covering of the denuded areas, making them difficult to detect [Racusen LC 1997]. It has been demonstrated in protracted ARF in humans that tubule epithelial cell damage results in trans-tubular backleak of glomerular ultrafiltrate, and that there is sluggish tubular fluid flow suggesting the existence of severe and generalised intraluminal tubular obstruction [Moran SM 1985].

The severity of renal injury that occurs following renal artery occlusion in the rat is dependent upon the ischaemic interval. When the duration of ischaemic injury is insufficient to cause necrosis, a variety of sublethal structural alterations occur [Molitoris BA 1992, Why SKV 1994]. These alterations may lead to impaired vectorial transport across the tubular epithelium and have been proposed to play a pathogenic role in causing sustained pre-glomerular vasoconstriction and filtration failure through tubuloglomerular feedback mechanisms [Alejandro V 1995, Kwon O 1998]. Sublethal cellular injury may therefore explain a well-recognised paradox of ischaemic acute renal failure whereby acute renal failure can occur in the presence of only relatively subtle histological changes [Racusen LC 1992].

In the rat model of complete renal artery occlusion the simultaneous presence of tubule epithelial cell damage, tubular obstruction and pre-glomerular vasoconstriction has made it difficult to assess the relative importance of each in the maintenance phase of renal failure. This has equally made it difficult to predict the sequence of events leading to recovery
Recovery from post-ischaemic ARF occurs in a biphasic pattern, the initial rise in glomerular filtration rate is associated with the relief of intratubular obstruction, with subsequent recovery occurring in association with progressive renal vasodilatation.

We have used a rat model of ischaemic ATN which is produced by 60 minutes of bilateral complete renal artery occlusion, followed by reperfusion [Basile DP 1996, Miller SB 1992, 1994]. In this model the straight portion of the proximal tubule (S3 segment) is most susceptible to ischaemic injury. The cells in this part of the nephron lose the normal tubular brush border and undergo extensive cell necrosis. Proximal tubule epithelial cells in the cortex (S1 and S2 segments) are less severly damaged. There is far less injury sustained to the cells lining the MTAL, with tubule epithelial cells in the inner medulla and those lining the collecting duct sustaining the least injury [Venkatachalam M 1978]. Functional studies have consistently demonstrated that the serum creatinine level peaks at 24 hours following surgery and thereafter declines as a function of time post-ischaemia. By seven days the mean serum creatinine values are no different between post-ischaemic and sham-operated controls. Figure 2.1 demonstrates the histological changes that occur in the outer medulla of the kidney following ischaemic renal injury. Figure 2.1A demonstrates the normal renal architecture in the outer medulla at low-power prior to ischaemia. At one day post-ischaemic renal injury sloughed necrotic cells are present in the lumens of tubules in the outer medulla (Figure 2.1B). It is particularly the straight portions of the proximal tubule (S3 segments, pars recta) that undergo the degree of most damage. By 7 days the tubules are markedly dilated and the cellular aggregates less prevalent (Figure 2.1C). Most tubules in the outer medulla are lined with simplified epithelial cells and hyperplastic papillary proliferative structures are evident (labelled "p"). By 14 days post injury the overall structure of the tubules present in the outer medulla is
improved (not shown). There is no morphological difference between the histological sections originating from kidneys of sham-operated rats versus those from unoperated rats.
Figure 2.1 Morphological characterisation of the outer renal medulla following acute ischaemic injury. Haematoxylin and eosin staining was performed on 5μm thick Bouins fixed sections that underwent sham surgery (A) or 60 minutes of bilateral renal artery ligation (B and C). Sections from rats rendered ischaemic are shown at 1 day post-injury (B) and 7 days post-injury (C).
2.2 Animals

The experimental protocols were conducted within the requirements of the Federal Government of USA regarding the humane care of laboratory animals and in adherence with the Declaration of Helsinki and to the principles of the American Physiological Society regarding the care of laboratory animals. In addition, all experimental protocols were performed with the prior approval of and in accordance with the guidelines laid down by the "Committee for the Humane Care of Laboratory Animals", Washington University, St Louis, Missouri, USA, which was the host institution.

Male Sprague-Dawley rats (Harlan, Indianapolis In) weighing 225-250 g were used. Rats were fed standard laboratory diet ad libitum and were housed in an animal facility at 21±2°C with a 12:12 hour light-dark cycle.

2.3 Induction of ischaemia

Ischaemic renal injury was induced by 60 minutes of bilateral renal artery clamping. Under anaesthesia induced by a combination of ketamine and pentobarbital sodium the abdominal cavity was exposed via a midline incision. Both renal arteries were identified and freed by blunt dissection. Microvascular clamps were placed on both renal arteries to affect complete cessation of blood flow. Core body temperature was maintained at 37±1°C by placing the animal on a homeothermic table and monitoring with a temperature-sensing rectal probe. After 60 minutes the clamps were removed with return of blood flow to the kidneys. If reperfusion was incomplete as judged visually the experiment was terminated and the animal killed. Sham-operated controls were included for each animal that underwent renal artery clamping. The abdominal cavity was exposed but no clamping of the renal arteries was performed.
2.4 **Measurements**

Animals were weighed daily. Tail vein blood was obtained before induction of ischaemic renal injury and then daily for seven days post surgery for measurement of creatinine and blood urea. Blood urea nitrogen and plasma creatinine were measured using standard colourimetric methods on a Multistat III plus autoanalyser (Instrumentation Laboratory Lexington, Massachusetts, USA). It has previously been demonstrated that the measurement of creatinine correlates well with the inulin clearance as a means to evaluate renal function post-ischaemic injury [Miller SB 1994].

2.5 **Isolation of kidney tissue**

At the prescribed times of each experiment the rats were anaesthetised with ketamine and pentobarbitol. Their kidneys were perfused with sterile phosphate buffered saline to remove all blood from the organs. The left kidney was quickly excised, frozen in liquid nitrogen and stored at -70°C. The right kidney was cut longitudinally into halves and stored in either Bouins or formaldehyde in preparation for immunohistochemical and in-situ hybridisation studies.

2.6 **Isolation of mRNA**

At the various time points indicated above, rats were anesthetized with ketamine and pentobarbital. Their kidneys were perfused with sterile phosphate-buffered saline (PBS) to remove all blood from the organs, quickly excised, frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated using the Ultraspec RNA isolation solution (Biotecx, Houston, TX). Tissue samples of rat kidney were homogenised with RNAzol B (2 ml per 100 mg tissue) with a few strokes in a glass-Teflon homogeniser. 0.2 ml chloroform per 2 ml of homogenate was added, and the samples were shaken vigorously for 15 seconds prior to incubating on the ice for 5 minutes. The suspension was centrifuged at 12,000 g at
4°C for 15 minutes. After centrifugation the homogenate formed two phases, with the RNA present in the upper aqueous phase. The upper aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added prior to storing the samples for 15 minutes on ice. The samples were then centrifuged for 15 minutes at 12,000 g (4°C) to precipitate the RNA as a white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet washed once with 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 7500 g (4°C). The RNA pellet was then allowed to air dry prior to solubilisation in diethylpyrocarbonate (DEPC) treated water. Total RNA was quantified using a spectrophotometer.

2.7 Acute renal failure cDNA library construction

An acute renal failure cDNA library was constructed from rat kidney. cDNA libraries represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then is inserted into a self-replicating lambda vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease. A representative cDNA library should contain full-length copies of the original population of mRNA.

The ZAP Express vector (Figure 2.2) allows both eukaryotic and prokaryotic expression, while increasing both cloning capacity and a number of unique lambda cloning sites. The ZAP Express vector has 12 unique cloning sites which will accommodate DNA inserts from 0 to 12 kb in length. Inserts cloned into the ZAP Express vector can be excised out of the phage in the form of the kanamycin-resistant pBK-CMV phagemid vector (Figure 2.3).
Clones in the ZAP Express vector can be screened with either DNA probes or antibody probes, and in vivo rapid excision of the pBK-CMV phagemid vector allows insert characterisation in a plasmid system. The polylinker of the pBK-CMV phagemid vector has 17 unique cloning sites flanked by T3 and T7 promoters and has three standardised primer sites for DNA sequencing. Transcripts made from the T3 and T7 promoters generate riboprobes useful for in-situ hybridisation. The lacZ promoter may be used to drive expression of fusion proteins suitable for Western blot analysis or protein purification.

Total RNA was isolated from kidneys obtained from rats 24 hours post-ischaemic injury using the Ultraspec RNA isolation solution (Biotecx, Houston, Texas). Poly A RNA was isolated by double passage over oligo-dT cellulose, and 5μg was used to synthesise double-stranded cDNA. The Zap Express cDNA synthesis kit uses a hybrid oligo (dT) linker-primer which contains an Xho I site. The restriction site allows the finished cDNA to be inserted into the ZAP Express vector in a sense orientation (EcoR I-Xho I) with respect to the lacZ promoter. First-strand synthesis was primed with the linker primer and was transcribed using Maloney mouse leukaemia virus reverse transcriptase (MMLV-RT) and 5-methyl dCTP. The poly (dT) region binds to the 3' poly (A) region of the mRNA template, and the reverse transcriptase begins to synthesise the first strand cDNA. The nucleotide mixture for the first strand contains normal dATP, dGTP and dTTP plus the analog 5-methyl dCTP. The complete first-strand has a methyl group on each cytosine base which protects the cDNA from restriction enzymes used in subsequent cloning steps.

During second strand synthesis, Rnase H nicks the RNA bound to the first-round cDNA to produce a multitude of fragments which serve as primers for DNA polymerase I. DNA polymerase I nick-translates these RNA fragments into second strand cDNA. The second
strand nucleotide mixture was supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. This ensures that the restriction site in the linker-primer will be susceptible to restriction enzyme digestion. The uneven termini of the double stranded cDNA were nibbled back or filled in with cloned pfu DNA polymerase, and EcoR I adapters were ligated to the blunt ends.

The Xho I digestion releases the EcoR I adapter and a residual linker primer from the 3' end of the cDNA. These two fragments were separated on a Sephacryl S-500 spin column. The size-fractionated cDNA was then precipitated and ligated to the ZAP Express vector arms.

The Lambda library was packaged in a high-efficiency system (Gigapack II Gold packaging extract) and plated on the E. coli cell line XL1-Blue MRF'. The library was then amplified to provide a large and stable quantity of high titre stock (1.3 x 10⁹ plaque forming units/ml).
Figure 2.2 Map of the ZAP Express vector.

Figure 2.3 Circular map of the pBK-CMV phagemid vector which allows the insert to be characterised in a plasmid system.
2.8 Differential display PCR

To identify those genes for which expression is upregulated post-ischaemic renal injury we used differential display-polymerase chain reaction (DD-PCR). This technology was developed by Drs. Pardee and Liang at Harvard Medical School [Liang P 1992] and represents a method for comparison, identification and isolation of genes expressed as mRNA in various cells or tissues under designated conditions. This method involves the reverse transcription of the mRNAs with oligo-dT primers anchored to the beginning of the poly A tail, followed by the PCR reaction in the presence of a second 13mer, arbitrary in sequence. The amplified cDNA of 3' termini of mRNAs as defined by this pair of primers are distributed on a DNA sequencing gel. By changing primer combinations, 15,000 individual mRNA species from a mammalian cell may be visualised. This provides fingerprinting for mRNA analogous to 2-D protein gel electrophoresis to visualise proteins. The analysis of mRNA samples side by side allow differentially expressed genes to be identified and probes to them to be recovered and used to clone their cDNAs or genomic DNA. Differential display-PCR has several technical advantages over methods such as a subtractive hybridisation. It is an extremely sensitive method requiring only 5μg of total RNA to cover all the anchored oligo-dT primers used in all combinations with 80 arbitrary 13mers. Statistically this would cover the majority of mRNAs in an eukaryotic cell. The technique is relatively simple, based on PCR and DNA sequencing gel electrophoresis and provides results relatively quickly.

It is crucial that the total RNA isolated is absolutely free of DNA contamination so that it can be used for the differential display PCR. MessageClean kit (GenHunter, Brookline MA) was used for the complete removal of any DNA contamination from the RNA samples. This kit uses DNase I, and a phenol/chloroform extraction procedure providing pure uncontaminated RNA. The RNA concentrations were determined using spectrophotometric readings at absorbance 260 nm and then stored at 1 to 2 μg aliquots at -70 °C prior to use.
Differential display-PCR was performed using an RNA image kit (GenHunter, Brookline MA). Deoxyribonuclease (Dnase) treated total RNA (0.2 ug) was reverse transcribed in a 20 ul reaction volume with Moloney mouse leukemia virus (MMLV) reverse transcriptase and the one base anchored primer T11-A. The complimentary DNAs (cDNAs) were then amplified by PCR in the presence of (α-35S) deoxy (d)-ATP (New England Nuclear, Boston MA) using the oligonucleotide primers T11A and 5'-AAGCTTAGCAGCA in a Perkin Elmer (Norwalk, CT) thermal cycler. Control reactions were performed in the absence of reverse transcriptase. Parameters for the PCR reactions were as follows: Denaturation at 94 °C for 30 sec; annealing at 40 °C for 2 min; and extension at 72 °C for 30 sec for 40 cycles followed by extension at 72 °C for 5 min. The amplification products were visualized following denaturing polyacrylamide gel electrophoresis and autoradiography. Amplification products ranging in size from 150-500 base pairs and showing repetitive differential expression in RNA derived from at least two sets of rats were reamplified and used for cloning, sequence analysis and as templates for random priming.

2.9 Cloning of PCR amplified fragments

Differential display-PCR products were cloned into the plasmid vector pCR-TRAP using the pCR-TRAP cloning system (GenHunter). This system utilises a positive selection strategy for selecting plasmids with PCR inserts. The vector contains a repressive gene which shuts off the promoter driving the tetracycline resistance gene also in the plasmid. Successful cloning of PCR products inactivates the repressive gene and turns on the tetracycline resistance gene. This allows the growth of clones containing the gene of interest. The presence of an insert in plasmid DNA was confirmed by amplifying the colony lysates by PCR using an oligonucleotide primer set that flanked the cloning site of the pCR-TRAP vector. The amplified inserts were gel-purified, radiolabelled and used as probes in RNA blot analysis. cDNA inserts that showed differential expression were sequenced from both directions using
the Aid Seq kit (GenHunter) in combination with sequenase kit 2.0 (U.S. Biochemical, Cleveland OH). The nucleotide sequences obtained were compared to known sequences by searching the GenBank and EMBL data bases with the BLAST algorithm.

2.10 Sequence analysis of PCR amplified fragments

Initially sequencing was performed using the sequenase kit 2.0 (U.S.Biochemical). This kit uses the chain-termination method which involves the synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded DNA template. The synthesis is initiated at only the one site where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analogue that will not support continued DNA elongation. The chain terminating nucleotide analogs are the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs). These lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme catalysed polymerisation will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information. A radioactively labelled nucleotide is also included in the synthesis, so the labelled chains of various lengths can be visualised by autoradiography after separation by high resolution electrophoresis.

At a later stage cDNA was completely sequenced by primer walking on an automated AB1 Prism 377 sequencing machine. Cycle sequencing was performed using a terminator ready reaction mix added to the cDNA template, primer and was made up to 20μl with dH₂O. The manufacturers recommended thermal cycling sequence was followed and the resulting extension products were purified prior to drying in a vacuum centrifuge. Using a 36 cm well-to-read gel up to approximately 600 bases of good data was provided per sequencing reaction.
2.11 Northern blot analysis

RNA obtained from kidneys of sham-operated rats and rats rendered ischemic was used in RNA blot hybridizations. Total RNA (20 μg) was fractionated in 2.2 M formaldehyde-1.2% agarose gels and transferred onto Zeta-Probe membranes (Bio-Rad, Hercules, CA) by RNA capillary transfer. Equal quantities of RNA were loaded onto each lane prior to electrophoresis as confirmed by analysis of 28S RNA bands. After transfer the membrane was separated from the gel and rinsed briefly in 2X SSC. The RNA was fixed onto the Zeta-Probe membrane using a UV linker. Prehybridisation was performed for one hour at 42°C followed by hybridisation with the specific cDNA probes overnight at 42°C using the recommended hybridisation solution. At the completion of hybridisation the membranes were rinsed at room temperature for 15 minutes in each of the following solutions: 2X SSC/0.1% SDS, 0.5X SSC/0.1% SDS, 0.1X SSC/0.1% SDS. After washing the blotted membranes they were exposed to x-ray film for varying times to obtain the optimal intensity pattern.

2.12 Radiolabelled cDNA probe construction

Specific probes were generated by labelling reamplified DD-PCR products or cloned full-length cDNA with [32P]dCTP (Amersham Corp., Arlington Heights, IL) using a random primer labelling kit (Stratagene, La Jolla, CA). This procedure relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The primer-template complexes formed represent a substrate for the klenow fragments of DNA polymerase I. The enzymes synthesise new DNA by incorporating nucleotide monophosphates at the free 3' OH group provided by the primer. The newly synthesised DNA is made radioactive by substituting a radiolabelled nucleotide [32P]dCTP for a nonradioactive one in their reaction mixture. Briefly, 25ng of cDNA template was mixed with 10μl of random oligonucleotide primers and made up to a total volume of 34μl with high-quality water. The reaction tubes were heated in boiling water for 5 minutes and then centrifuged at
room temperature. Next the following reagents were added to the reaction tubes: 10μl of 5X primer buffer, 5μl of [32P]dCTP and 1μl of Exo (-) Klenow enzyme. The reaction components were mixed thoroughly and incubated at 37-40°C for 10 minutes. Removal of unincorporated nucleotides was achieved using Stratagene's Nuc Trap probe purification columns.

2.13 Full length cDNA cloning and sequence analysis

The acute renal failure cDNA library was screened using the cloned DD-PCR product. The cDNA library was plated onto 150 mm NZY plates with NZY top agar and incubated at 37°C overnight. The resulting cDNA plaques were transferred to a nitrocellulose membrane, which was denatured using a 1.5 M NaCl and 0.5 M NaOH denaturation solution for 2 minutes. The nitrocellulose membrane was neutralised for 5 minutes by submerging in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralisation solution. The nitrocellulose membrane was then rinsed for 30 seconds by submerging in a 0.2 M Tris-HCl (pH 7.5) and 2X SSC buffer solution. The cDNA was then UV cross-linked to the membranes. Prehybridisation of the membrane was performed at 42°C overnight using the recommended prehybridisation solution. The cloned DD-PCR product was radiolabelled with fresh [α-32P] dATP using the Prime-It II random primer labelling kit (Stratagene), and hybridisation performed overnight with the recommended hybridisation solution. Following hybridisation the nitrocellulose membrane was washed using a 0.1X SSC buffer and 0.1% SDS wash solution at 65°C with shaking. After washing, the membrane was placed between two sheets of plastic wrap in a cassette with intensifying screens at -80°C. Several positive clones were isolated to purity and the pBK-CMV plasmids containing the inserts were rescued from the Zap Express vector by in vivo excision with the ExAssist/SOLR system (Stratagene). Plasmid DNA containing the inserts was prepared using the magic mini or maxi DNA purification systems (Promega,
Sequence analysis was carried out using T3, T7 and specific oligonucleotides in combination with the sequenase kit 2.0 (U.S. Biochemical).

2.14 In-situ hybridisation

In-situ hybridisation was performed on tissue sections originating in kidneys from sham-operated rats and rats rendered ischaemic. Kidney tissue was fixed by immersion (to eliminate variation in perfusion efficacy between sham and post-ischaemic kidneys) in Bouin's solution overnight, washed in 70% ethanol, and embedded in paraffin. Five-micrometre sections were deparaffinised in xylene, gradually rehydrated in decreasing concentrations of ethanol and pretreated with 1μg/ml proteinase K (Boehringer Mannheim, Indianapolis IN) in 0.01 M Tris, pH 8.0, containing 50 mM EDTA for 30 minutes at 37°C. After termination of the reaction in 0.01 M Tris, pH 7.4, containing 0.1 M glycine, the sections were further treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 minutes, dehydrated through a graded series of ethanol, and air dried. Digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis IN) labelled antisense probes were generated from the relevant cDNA using the appropriate RNA polymerase. As a control, sense-strand RNA corresponding to the same region as the antisense probe was synthesised in the opposite direction using the appropriate RNA polymerase. The size, integrity and quantity of probes were verified by gel electrophoresis and ethidium bromide staining. After heating the probes to 80°C for 5 minutes, hybridisation was performed overnight at 50°C in a humid chamber with a probe concentration of 1ng/μl in 20 mM Tris, pH 8.0, containing 50% formamide, 0.3 M NaCl, 1mM EDTA, 1X Denhardt's solution (Sigma), 80μg/ml denatured salmon sperm DNA (Sigma), 500μg/ml yeast tRNA (BRL, Bethesda, MD), and 10% dextran sulphate. After hybridisation, cover slips were removed in 4X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and washed in the same buffer for 90 minutes at 60°C followed by washing in 0.1 X SSC with 50% formamide at 50°C. The sections were further treated with
Rnase T1 (1 U/ml, Boehringer Mannheim) for 30 minutes, washed again in 0.1 X SSC at 37°C. Sections were blocked with 2% normal sheep serum and incubated with the anti-digoxigenin antibody (Boehringer Mannheim). Colour development was with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylyl phosphate in the presence of levamisole and the reaction was stopped when staining was judged to be satisfactory. The tissues were mounted in aqueous medium and photographed under light microscopy.

2.15 Immunohistochemistry

Immunohistochemistry was performed on 5 µm Bouin's-fixed paraffin embedded sections. The tissue sections were deparaffinisation in xylene for 15 minutes and rehydrated through graded concentrations of ethanol. When necessary, antigen presentation was enhanced by microwaving the sections whilst immersed in 1M sodium bicarbonate pH 6.0 for 15 minutes. The slides were then allowed to cool for 30 minutes prior to washing twice in PBS for 5 minutes. The tissue was then pretreated with 0.6% H₂O₂ in 80% methanol for 15 minutes, followed by three washes in PBS for 5 minutes each. Excess PBS was drained from the slides and the tissue sections were circled using a hydrophobic Pap pen. Sequential incubations were carried out in solutions containing avidin and biotin (Zymed, San Francisco, CA) respectively for 30 minutes each in a humid chamber, followed by 3 washes in PBS for 5 minutes each. The tissues were blocked for 1 hour in blocking buffer (0.01M PBS, pH 7.4, containing 0.3% Triton X-100, 10% normal goat serum, 0.3% BSA, and 100 mg/ml goat γ-globulin) in a humid chamber at room temperature. The primary antibodies were applied in the blocking buffer at concentrations ranging from 1: 100 to 1: 1000 for 1 hour at room temperature or overnight at 4°C in a humid chamber. The slides were then washed three times in PBS prior to the addition of the species-specific biotinylated secondary antibody for 1 hour at room temperature in a humid chamber. Detection was performed using a streptavidin-biotin immunoperoxidase technique with aminoethylcarbozol as a substrate (Histostain SP kit,
Zymed, San Francisco CA). The slides were then washed for 15 minutes in deionised water and counterstained with haematoxylin for 2 minutes. Mounting of the slides was performed using GVA mounting solution. The specificity of staining was verified by performing control experiments in which the appropriate species-derived IgG and normal serum was substituted for the primary antibody.

As described above antigen unmasking or retrieval was performed in some cases. Fixation of tissue has been recognised to induce masking of many antigens of diagnostic or prognostic value [Catteretti G 1995]. The type and length of fixation have an important influence on the extent of antigen masking. We effectively employed the use of microwave oven heating of slides for 15 minutes submerged in 1M sodium bicarbonate pH 6.0 when indicated.

2.16 Reverse transcription-PCR

Total RNA was extracted and treated with deoxyribonuclease, as described previously, to render it free of DNA. RNA concentrations were determined using spectrophotometric readings at absorbance 260 nm. One microgram of RNA was reverse transcribed at 42°C for 15 minutes in the presence of 10X first strand buffer, 1 mmol/l dNTP, 20 U Rnasin, 2.5 μM random hexamers and 500 U of Maloney murine leukaemia virus reverse transcriptase (Superscripts, Gibco BRL) in a 20 μl reaction volume. First-strand cDNA (10 μl of RT reaction mix) was amplified using 2.5 U Taq polymerase (Perkin Elmer, Foster City, CA, USA) in a 40 μl reaction volume containing 0.1 μmol/l primer pair and 10X buffer. The amplifying conditions were adjusted to optimise annealing conditions for specific primer pairs. The PCR products were analysed in a 1% agarose gel stained with ethidium bromide. Agarose gel extraction was performed using the QIAEX II gel extraction kit (Qiagen, Valencia, CA, USA). During this procedure all non-nucleic acid impurities such as agarose,
proteins, salts, and ethidium bromide are removed. Extracted cDNA was stored at - 20°C for further use.

2.17 Differential library screening

A small fraction of the acute renal failure cDNA library generated from kidneys of adult rats sacrificed 24 hours post-ischaemic injury was plated on a Nunc bioassay dish (Fisher, Pittsburg, PA, USA). Replica filters were made from the plates on zeta-probe membranes (BioRad, Hercules, California, USA). Total RNA was isolated from kidneys of sham-operated rats and rats rendered ischaemic at 24 hours post-surgery. For differential library screening, first-strand 32P-labelled cDNA was synthesised from 5μg of total RNA using superscript reverse transcriptase (BRL, Bethesda, Maryland, USA). One of the replica filters was then hybridised to the cDNA probe from a sham-operated kidney, and the second filter was hybridised to the probe from ischaemic kidney. The hybridisation and washing conditions were those recommended by the manufacturer of the zeta-probe. The hybridised filters were then exposed to x-ray films for two days at - 70°C and the autoradiograms aligned with each other. The plaques that exhibited differential hybridisation were isolated and their differential expression confirmed by northern blot hybridisation.

2.18 In-situ detection of apoptosis

Detection of apoptosis was performed by using the ApopTag in-situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD). This kit detects apoptotic cells by direct immunoperoxidase detection of digoxigenin-labelled genomic DNA in thin sections of fixed tissue.
Cell death through apoptosis follows a multistage process of DNA fragmentation. Large
fragments of 300 kb and 50 kb are first produced by endonucleolytic degradation of higher
order chromatin structural organisation. Further cleavage by endonucleases occurs at
linker DNA sites between nucleosomes. This results in DNA fragments that are multimers
of about 180 base pairs and the characteristic appearance of "DNA laddering" when
samples are run on a standard agarose gel. The ApopTag kit uses terminal
deoxyribonucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL) to
label the multitude of new 3'-OH DNA ends generated by DNA fragmentation. Residues of
digoxigenin-nucleotide are catalytically added to the DNA by TdT. The incorporated
nucleotides form a random heteropolymer of digoxigenin-11-dUTP and dATP, in a ratio
that has been optimised for anti-digoxigenin antibody binding. The anti-digoxigenin
antibody fragment carries a conjugated reporter enzyme (peroxidase) to the reaction site.
The localised peroxidase enzyme then catalytically generates an intense signal from
chromogenic substrates. Cells exhibiting necrotic morphologies can in some instances
contain stainable concentrations of DNA ends, but they appeared more diffuse than
apoptoses.

Paraffin-embedded tissue sections were deparaffinised in xylene for 15 minutes and
rehydrated through graded concentrations of ethanol. After washing with PBS three
times for five minutes the sections were circled using a hydrophobic Pap pen and treated with
20µg/ml proteinase K in PBS at 37°C for 15 minutes and washed with deionised water for
10 minutes. To inactivate endogenous peroxidase the tissue sections were incubated in 2%
hydrogen peroxide in PBS for 15 minutes at room temperature, and then rinsed twice in
PBS, for five minutes each time. The slides were then incubated with a TdT buffer
(25mmol/l Tris-HCl buffer, pH 6.6, 0.2mmol/l potassium cacodylate, and 0.25mg/ml BSA)
at room temperature for 30 minutes. Next the slides were reacted with 0.1U/µl TdT
dissolved in a TdT buffer supplemented with 1.0nmol/l digoxigenin-dUTP in a humid chamber at 37°C for 1 hour and then washed in PBS three times for five minutes each wash. Horseradish peroxidase-conjugated sheep anti-digoxigenin antibody was added for 30 minutes at room temperature in a humid chamber. The slides were washed three times in PBS for 5 minutes each wash and colour development performed through the addition of freshly filtered 0.05% diaminobenzidine (DAB) and 0.02% hydrogen peroxide for 3 to 6 minutes at room temperature. The slides were then washed in deionised water for five minutes prior to mounting in GVA mounting solution. Negative control sections were also included with sham staining performed by substituting deionised water for TdT enzyme.

2.19 Administration of PARP inhibitors to rats

The PARP inhibitors benzamide and 3-amino benzamide were purchased from Sigma Chemicals (St Louis Missouri). The inhibitors were dissolved in saline at a concentration of 16 mg/ml for benzamide and 5 mg/ml for the 3-amino benzamide.

Benzamide (40 mg/kg) or 3-amino benzamide (10 mg/kg) was administered intraperitoneally every 8 hours for a 24 hour time period immediately post-ischaemic injury (4 doses). Vehicle-treated rats received four saline injections instead. Tail vein blood was collected before induction of ischaemic injury and at every 24 hours post-ischaemia. Recovery from ischaemic injury was monitored by evaluating the levels of creatinine and blood urea nitrogen (BUN) as previously described various time points following the treatment. In control experiments, it was determined that the administration of benzamide or 3-amino benzamide to sham-operated rats in this manner had no effect on levels of creatinine or BUN measure daily for six days post injury. Statistical analysis was performed using the ANOVA-Bonferroni multiple comparison test.

2.20 PCNA analysis
Nuclear proliferating cell nuclear antigen (PCNA) was detected using a PCNA staining kit (Zymed, San Francisco, CA). The kit utilizes a biotynylated PCNA monoclonal antibody. Colour development was with the aminoethyl carbozol (AEC) staining kit (Zymed). Formalin fixed kidney sections from vehicle or PARP inhibitor treated rats were utilized in the study. Eight kidney sections originating from four different rats were viewed under X10 power lens, and the images from non overlapping outer medullary (S3) segments were captured on a computer. The purple-coloured nuclei were counted using the Image-Pro plus software (Media Cybernetics, Silver Spring, MD). Data were expressed as the number of regenerating cells per square millimetre. To avoid potential error in statistical sampling 20-25 non-overlapping, 1 square-millimetre fields were randomly selected and counted in a blinded manner. Statistical analysis was done using Student's t-test.

2.2.1 Western Blotting

Whole kidneys from rats were homogenised in cold buffer (20 mM Tris-HCL, pH 7.5, 250 mM sucrose, 10mM EDTA, 10 mM PMSF, 20 μg/ml leupeptin, 1% Nonidet P40). Homogenates were centrifuged at 1000 X g for ten minutes at 4°C to separate the soluble (cytosolic) fraction from the membrane fraction. The membrane fraction was resuspended to half the original volume using in the homogenisation buffer to which Triton X-100 was added to a final concentration of 0.3%. Twenty micrograms of protein was fractionated on a 4 to 20% gradient polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were incubated in 3% non-fat milk in Tris-buffered saline containing 0.05% Triton X-100 for one hour, then at 4° C overnight with, the specific primary antibody in Tris-buffered saline. The membranes were washed and incubated with horse radish peroxidase conjugated anti-goat antibody (Pierce, Rockford, Illinois, USA) for one hour at room temperature. The reaction products were visualised using ECL-chemiluminescence kit (Amersham, Arlington Heights, Illinois, USA).
2.22 Quantitation of tubular dilatation

The degree of morphological damage was assessed using sections of kidneys stained with haematoxylin and eosin. The number of dilated tubules from 15 to 20 non-overlapping fields in the outer medullary segments of both kidneys was determined by selecting tubules with a diameter of > 20µm using light microscopy and the Image-pro plus software. The data were expressed as the number of dilated tubules per square millimetre.

2.23 Measurement of ATP content

To determine the ATP content, kidneys were snap frozen in liquid nitrogen (< 1 second) and homogenised in 2% trichloro acetic acid. A small aliquot of the lysate was neutralised with 0.1M Tris buffer (pH 9.0), and serial dilutions were made. The diluent was mixed with 100 µl of luciferase-luciferin reagent (Promega, Madison, WI), and luminescence was detected with a 5 second time delay and a 10 second signal integration time in a Zylux luminometer (Zylux, Maryville, TN). A five-point ATP standard curve was generated using known concentrations of ATP and the corresponding luminescence. The quantity of ATP in the lysate was determined by performing a linear regression on the data from the ATP standard curve. Three animals were used from each group, and ATP assay was performed on samples from both kidneys. Statistical analysis was done using Dunnett's multiple-comparison test.

2.24 Statistics

All results in this thesis are expressed as mean ± standard error of the mean. Statistical analysis was performed using the unpaired Student's t-test or Dunnett's multiple-comparison test. To compare values between multiple groups analysis of variance (ANOVA) was used. A P value <0.05 was considered statistically significant.
CHAPTER 3

INDUCTION OF CALCYCLIN AFTER ISCHAEMIC INJURY TO RAT KIDNEY

3.1 Introduction

In contrast to ischaemic injury of the brain, the kidney has the ability to restore structure and function completely. Following ischaemic renal injury cell death occurs predominantly in the proximal tubules lying in the outer medulla and inner cortex. The recovery of renal function is therefore dependent on regeneration of proximal tubule structure and function. Proliferating cell nuclear antigen PCNA a cDNA polymerase δ-associated protein is a marker for the G1-S transition in the cell cycle and hence mitogenesis. Previous work has demonstrated that following renal ischaemic injury PCNA is detectable in the nuclei of epithelial cells of the S3 segments of the proximal tubules [Witzgall R 1994]. In the model of ischaemic renal injury that we have used we have confirmed these findings with proliferation being maximal at 24-48 hours. The same cell population that is undergoing proliferation also expresses the mesenchymal marker vimentin, indicating that these cells are undergoing de-differentiation. During this process the remaining viable tubule epithelial cells undergo de-differentiation and mitogenesis, progressing through the cell cycle to restore epithelial integrity and cell function [Bonventre JV 2003]. These events are regulated by the orchestrated expression of a number of renal genes including those coding for transcription factors, structural proteins and growth factors [Safirstein R 1990]. The re-establishment of cellular function also requires synthesis of new extra-cellular matrix and reformation of cell-cell contacts. Differential gene expression occurs immediately following ischaemic injury and plays an important role during the recovery phase.
Higher organisms contain about 100,000 different genes of which perhaps only 15% are expressed in any one individual cell. It is ultimately the choice of which genes are expressed that determines all life processes. Differential gene expression is fundamental for development and differentiation, homeostasis, response to insults, cell cycle regulation and cell death and lies at the heart of the regulatory mechanisms that control cell biology. The ability to compare gene expression in different cell types in response to pre-determined conditions could provide us with essential information to enable the analysis of the biological processes that underlie disease processes, such as ischaemic acute renal failure.

Following renal ischaemic injury, several groups have observed the rapid and transient induction of several early response genes [Safirstein R 1994]. It has been speculated that renal tubule epithelial cell injury and necrosis initiate a proliferative regenerative programme dependent on differential gene expression.

Using differential display-polymerase chain reaction (DD-PCR) we identified the enhanced expression of calcyclin post-ischaemic renal injury. Calcyclin, also known as S100 A6, is a member of the S100 family of calcium-binding proteins [Zimmer DB 1995]. These proteins are characterized by the possession of highly conserved helix-loop-helix calcium-binding domains known as EF hand motifs. The S100 proteins have been implicated in cell cycle regulation and differentiation as effectors in calcium mediated signal transduction pathways. The increased expression occurred as early as 6 hours after ischaemia and is demonstrable not only in the regenerating proximal tubule, but also in the thick ascending limb of Henle and the distal tubule. These findings are consistent with increased calcyclin expression of playing a role in the regenerative processes following ischaemic renal injury.
3.2 Methods

Ischaemia was induced in male Sprague-Dawley rats (Harlan, Indianapolis, IN) for 60 minutes, as described in Chapter 2. To control for the extent of renal injury during the procedure, animals were selected such that the level of serum creatinine measured 24 hours following injury fell into the range used in previous studies [Padanilum BP 1996]. At 24 hours serum creatinine levels (mean ± SE, n= 3 rats) were 4.2 ± 0.3; 3.2 ± 0.5; 3.0 ± 0.3; 3.5 ± 0.1; and 3.2 ± 0.4 mg/dl in groups of rats used to obtain kidneys 1, 3, 5, 7 and 14 days post-ischaemia respectively (Table 3.1). Creatinine levels were 0.8 ± 0.1; and 2.6 ± 0.1 mg/dl (n = 3 rats), in the groups of rats sacrificed at 6 and 12 hours post-injury (Table 3.2). Levels of serum creatinine in sham-operated rats were measured at the time of sacrifice and averaged 0.6 ± 0.01 mg/dl (n=21). These levels were not significantly different between groups.

<table>
<thead>
<tr>
<th>No. of days post-ischaemia</th>
<th>Serum creatinine at 24 hours (mg/dl)</th>
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<tr>
<td>1</td>
<td>4.2± 0.3</td>
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<tr>
<td>3</td>
<td>3.2± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>3.0± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>3.5± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>3.2± 0.4</td>
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</table>

Table 3.1 Serum creatinine levels (mg/dl) at 24 hours in groups of rats used to obtain kidneys 1,3,5,7 and 14 days following ischaemic renal injury.
Table 3.2 Serum creatinine levels (mg/dl) in the groups of rats sacrificed at 6 and 12 hours post-ischaemic injury.

Levels of serum creatinine at the time of sacrifice of rats that had been rendered ischaemic were 4.2 ± 0.3; 1.9 ± 0.9; 0.8 ± 0.1; 0.7 ± 0.1; and 0.6 ± 0.1 mg/dl at 1, 3, 5, 7, and 14 days post-injury respectively (Table 3.3).

<table>
<thead>
<tr>
<th>No. of days post-ischaemia</th>
<th>Serum creatinine (mg/dl) at the time of sacrifice</th>
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<tr>
<td>1</td>
<td>4.2± 0.3</td>
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<tr>
<td>3</td>
<td>1.9± 0.9</td>
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<tr>
<td>5</td>
<td>0.8± 0.1</td>
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<td>7</td>
<td>0.7± 0.1</td>
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<tr>
<td>14</td>
<td>0.6± 0.1</td>
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Table 3.3 Serum creatinine levels at the time of sacrifice of rats that had been rendered ischaemic at 1,3,5,7 and 14 days.
3.2.1 **Differential display PCR**

DD-PCR was performed using a kit (Gen Hunter, Brookline MA) as previously described in Chapter 2.

3.2.2 **Northern blot analysis**

RNA blot hybridisations were performed as previously described in Chapter 2. Equal quantities of RNA were loaded onto each lane prior to electrophoresis as confirmed by analysis of 28S RNA bands. The blots were exposed to X-ray film for varying times to obtain the optimal intensity pattern.

To quantify calcyclin mRNA, samples from 3 animals/group and 3 corresponding sham-operated control animals were analysed by northern blotting and band intensity measured using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Comparison between gels was accomplished by normalizing data to the value of the respective band originating from a kidney of a sham-operated rat.

Statistical analysis was performed using the Student's t-test for unpaired sample means. P < 0.05 for 2-tailed analysis was considered significant.

3.2.3 **Cloning and sequence analysis of PCR amplified fragments**

DD-PCR products were cloned into the plasmid vector pCR-TRAP using the pCR-TRAP cloning system (GenHunter). The nucleotide sequences obtained were compared to known sequences by searching the GenBank and EMBL data bases with the BLAST algorithm.
3.2.4 Full length cDNA cloning and sequence analysis

A cDNA library was generated using poly A RNA from kidneys of adult rats rendered ischaemic 24 hours previously in the Lambda Zap Express vector (Stratagene). The library was screened using the cloned DD-PCR product as described previously in Chapter 2. Several positive clones were isolated to purity and the pBK-CMV plasmids containing the inserts were rescued from the Zap Express vector by in vivo excision with the ExAssist/SOLR system (Stratagene). Plasmid DNA containing the inserts was prepared using the magic mini or maxi DNA purification systems (Promega, Madison WI). Sequence analysis was carried out using T3, T7 and specific oligonucleotides directed to the calcyclin sequences in combination with the sequenase kit 2.0 (U.S.Biochemical).

3.2.5 In-Situ Hybridisation

The full length calcyclin cDNA in plasmid pBK-CMV was used as the template to generate Digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis IN) labelled sense and antisense probes.

3.2.6 Immunohistochemistry

Localisation of calcyclin was performed on Bouin's-fixed paraffin embedded sections as previously described. The primary calcyclin antibodies were obtained from Swant Laboratories, Switzerland. Antigen presentation was enhanced by microwaving the sections. The primary calcyclin antibody was applied in the blocking buffer at 1:100 concentration for 1 hour at room temperature. Detection was performed using a streptavidin-biotin immunoperoxidase technique with aminoethylcarbozol as a substrate (Histostain SP kit, Zymed, San Francisco CA). The specificity of staining was verified by performing control experiments in which rabbit IgG and normal rabbit serum was substituted for anti-calcyclin. Positive staining was not observed in control experiments.
3.3 Results

Differential gene expression was investigated using total RNA obtained from renal tissue of either sham-operated rats or rats that had been rendered ischaemic 1 day previously (Figure 3.1). Isolation of differentially expressed genes was performed by excision of corresponding bands from the DD-PCR gel. The bands were re-amplified, radiolabelled and used as a probe in northern analysis to confirm differential expression. The plasmid pCR-TRAP system was used to clone the re-amplified product. Further northern analysis confirmed the successful cloning of the differentially expressed gene. The plasmid DNA was purified and then sequenced using oligonucleotide primers flanking the insertion site of the vector. Initial sequencing identified a 300 base pair fragment which showed some homology with calcyclin. To obtain the full length cDNA it was necessary to screen an acute renal failure cDNA library generated from rat kidney. Several positive clones were selected containing the full length sequence for calcyclin which was confirmed after submission to the Genbank.

The time course of calcyclin expression was characterized post-ischaemic injury using sham-operated rats as controls. Northern assays were performed using RNA from kidneys at 6 and 12 hours and 1, 3, 5, 7 and 14 days post-sham surgery or post induction of ischaemic injury. Shown in figure 3.2 are Northern blots of RNA extracted from kidneys of ischaemic rats (acute renal failure, ARF) and rats subjected to sham surgery from 1 and 3 days post event. Calcyclin mRNA is demonstrable as a single 0.5-kb band, as would be expected [Calabretta B 1986]. Levels can be seen to be enhanced following ischaemic injury.
Figure 3.1 Differential display-polymerase chain reaction (DD-PCR): representative gel run with RNA derived from kidneys of 2 rats. Lane 1, DD-PCR products from a sham-operated rat; and Lane 2, from a rat rendered ischaemic 1 day previously (acute renal failure, ARF). One differentially expressed band (calcyclin) is shown by an arrow.
Figure 3.2 Northern assay for calycyclin mRNA. Top: levels of calycyclin mRNA extracted from kidneys originating from sham-operated rats all rendered ischaemic (ARF) at 1 (1D) or 3 days (3D) post-surgery: representative autoradiogram of a Northern blot. Size of the RNA species (0.5kb) is shown at right. Bottom: autoradiogram of the same RNA samples rehybridised with a radiolabelled probe for 28S RNA, demonstrating consistent levels of 28S RNA.
Levels of calcyclin mRNA in kidneys were quantified using a phosphorimager. Levels were elevated 2.4-fold in kidneys originating from ischaemic rats relative to those in kidneys of sham-operated controls as early as 6 hours post-ischaemia (P = 0.05, Student's t-test). Levels were elevated approximately 10-fold at 1 day following injury (P = 0.0005). Thereafter, levels declined but remained elevated at 3 days (P = 0.003), 5 days (P = 0.009), 7 days (P = 0.009) and 14 days post-ischaemia (P = 0.01) (Figure 3.3).
Figure 3.3 Effect of ischaemia on renal calcyclin mRNA levels. Results from three Northern assays performed as in Figure 2 were quantified using a phosphorimager. Data are means ± SE. Levels were significantly elevated as early as 6 hours following ischaemic injury (see results section).
In-situ hybridisation was performed to localize the expression of the calcyclin transcript (Figure 3.4). There was no calcyclin mRNA expression identified in the glomeruli, proximal tubules or thick ascending limbs of Henle 1 day following sham surgery or thereafter (not shown). However, calcyclin mRNA was detected in cells lining the renal pelvis and calyces (not shown). At 1 day post-ischaemic injury, calcyclin mRNA was detected in cells in the outer medulla (Figure 3.4, top right). Higher-power views demonstrate that both the epithelium of damaged proximal tubules that contain sloughed epithelial cells (Figure 3.4, middle left) and the thick ascending limb of Henle (Figure 3.4, middle right) stain positively for calcyclin mRNA. By 3 days post-ischemia calcyclin mRNA was still expressed in the thick ascending limb (not shown) and was evident in damaged proximal tubules in the outer medulla (Figure 3.4, bottom right). The use of a sense control probe showed little or no hybridisation. Calcyclin mRNA expression was reduced by 7 days post injury but could still be seen in the thick ascending limb (not shown).
Figure 3.4 Localisation of renal calsecynin mRNA by non-isotopic in-situ hybridisation. Calcycin mRNA in kidneys from rats subjected to sham surgery (1S) or rendered ischaemic (1 ARF) 1 day prior to death or in kidneys of rats rendered ischaemic 3 days prior to death (3 ARF) was localised using a digoxigenin labelled antisense riboprobe. Arrowhead, cells lining damaged proximal tubules (1 ARF, middle left). Arrow, medullary thick ascending limb of Henle's loop (1 ARF, middle right). 3 ARF-C, control, sense probe.
The distribution of peptide post-injury was demonstrated using anti-calcyclin antibody (Figure 3.5). Immunohistochemical staining of sham-operated rats 1 day post surgery localised peptide in the glomeruli, distal tubules (DT), and in cells lining the renal pelvis and calyces (not shown). There was no expression in the thick ascending limbs of Henle. In comparison after the induction of ischaemic injury peptide was markedly up-regulated in the medulla (Figure 3.5, top right) predominantly in the thick ascending limb of Henle. In the cortex calcyclin peptide was also up regulated in distal tubules. The numbers of positively stained distal tubules were counted in 10 comparably located microscopic fields of sections of cortex originating from five sham-operated and five post-ischaemic rats. There was a 3.5-fold increase in the number of distal tubules displaying the peptide post-ischaemia compared with the post sham surgery (19.7± 0.69 versus 5.7± 2.0, means±SE; P < 0.0001, Student's t-test). Calcyclin was also expressed in some but not all dilated proximal tubules in the renal cortex (arrowhead). At 3 days post-ischaemia calcyclin protein could be seen in the damaged S3 segments of the proximal tubules in the outer medulla (3 ARF). The protein expression was reduced by 7 days (not shown).
Figure 3.5 Immunohistochemical localisation of calcyclin peptide in kidneys. Calcyclin peptide in kidneys from rats subjected to sham surgery (S) or rendered ischaemic (ARF) 1 or 3 days prior to death was localised using an antibody to calcyclin. Arrowheads, cells lining damaged proximal tubules. Arrow, thick ascending limb of Henle. G, glomerulus; DT, distal tubule. Sections are representative of > five experiments.
3.4 Discussion

Calcyclin is a member of the S100 family of EF-hand calcium binding proteins which has grown to be one of the largest sub families of EF proteins [Zimmer DB 1995]. In common with other members of this family it is a low molecular weight (10-12KDa) acidic protein, which is highly enriched in nervous tissue. Characteristically they possess highly conserved helix-loop-helix calcium binding domains known as EF-hand motifs [Krebs J 1995], flanked by hydrophobic regions at either terminus. The carboxy-terminal of EF hand proteins encompasses 12 amino acids whereas the amino-terminal loop is formed of 14 amino acids and has a lower affinity for calcium ions. There are 17 different proteins currently assigned to the S100 family with calcyclin denoted by S100A6. They show different degrees of homology ranging from 25 to 65% identity at the amino acid level. The genes for at least ten of the S100 proteins are situated in a tightly clustered locus on human chromosome 1 (1q21), a region frequently involved in chromosomal translocations in human breast cancer [Schafer BW 1996]. The physical and structural properties of S100 proteins suggest that they play a dynamic role as activating proteins rather than simply acting as buffers.

Calcium ions act as second messengers to control many biological processes through the interaction with calcium binding proteins. Intracellular calcium concentration is known to control a wide variety of cellular processes such as cell cycle progression, differentiation, muscle contraction and enzyme activities. Calcium binding proteins act as intracellular calcium receptor molecules and couple changes in intracellular calcium to alterations in cell function. Altered intracellular calcium levels are linked to various disease states including ischaemic renal injury [Schafer BW 1996]. It is therefore important that intracellular calcium levels and calcium signalling are tightly controlled. Activating ligands such as growth factors bind to membrane receptors resulting in a rise of intracellular calcium concentrations. The
binding of Ca\textsuperscript{2+} to S100 proteins leads to a conformational change exposing the hydrophobic regions in the molecule and allows target protein interaction [Kligman D 1988].

Individual S100 proteins show specific patterns of tissue and cellular distribution, which contrasts with the ubiquitous expression of the calcium binding protein calmodulin. They have been implicated in the control of a variety of cellular functions including cell growth and differentiation, cytoskeletal function and cell structure, energy metabolism and intracellular signal transduction pathways [Zimmer DB 1995]. Most of the S100 proteins function intracellularly, however despite lacking a classical leader peptide, some are secreted. The S100B dimer can act as a glial mitogen and as a neurotropic factor, stimulating neurite outgrowth and/or survival of specific neuronal populations. Interestingly S100B can act as a growth or differentiation factor at lower concentration or induce apoptosis at higher concentrations [Schafer BW 1996].

Calcyclin was first identified as a cell cycle specific cDNA that was preferentially expressed in the G1 phase of the cell cycle in a temperature sensitive hamster cell line [Hirschhorn RR 1984]. It was later shown that calcyclin mRNA levels are increased in response to serum, PDGF and EGF in quiescent fibroblasts [Calabretta B 1986]. Its expression in quiescent cells following mitogenic stimuli may result from the activation of the protein kinase C system [Gong Y 1992]. These characteristics have implicated calcyclin in the regulation of cell growth and proliferation. Further work has demonstrated increased and deregulated calcyclin expression in the cells of patients with acute myeloid leukemia, and in certain human breast cancer cell lines [Potts BCM 1995]. The role for calcyclin in cell division is suggested by its ability to bind to the regulatory domain of annexin XI [Tokumitsu H 1992]. Annexin XI localizes to the nuclei of certain cells, and its concentration around the mitotic apparatus during M-phase implicate it in the regulation of cell growth or division [Mitzutani A 1992].

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More recently the three dimensional structure of calcyclin has been described [Potts BCM 1995] which reveals a symmetric homodimeric fold that is unique among calcium binding proteins.

Our results in rat, and data derived on human tissues [Kuznicki J 1992] indicate that calcyclin peptide is normally present in renal glomeruli, some distal tubules (Figure 3.5), and cells lining the renal pelvis and calyces (not shown) convoluted tubules and the transitional epithelium of the renal pelvis. In-situ hybridisation fails to demonstrate localised calcyclin mRNA to these sites, consistent with the findings of another group who found that the only cells in mouse kidney in which calcyclin mRNA could be found were those lining the renal pelvis and calyces [Timmons PM 1993]. This lack of direct correlation between mRNA and protein levels in rat tissues has also been previously noted [Zimmer DB 1991]. This suggests that there is little calcyclin produced in non ischaemic renal parenchyma, and the peptide that is present represents calcyclin accumulated from extracellular sources or that the variability of S100 gene expression may be secondary to cell specific differences in mRNA transcription, or protein stability. It is unknown what normal physiological function calcyclin serves in these cells.

The timing of calcyclin expression in the S3 segment of proximal tubules post-ischaemic injury coincides with the period of maximal cellular regeneration and proliferation (2-3 days post injury). Thus levels of renal calcyclin are increased by 6 hours post injury, the peak of expression occurs at one-day following ischaemia, and the increased levels persist for up to three days. This implies that calcyclin is playing an important role during the regenerative phase. Failure to demonstrate calcyclin peptide in all damaged cortical proximal tubules probably reflects differences in the timing and extent of proximal tubule injury and regeneration.
The finding of increased expression of calcyclin transcript and peptide in the thick ascending limb of Henle and the distal tubule is intriguing. These cells are removed from the site of greatest injury, however, it is known that they undergo both positive and negative gene transcription post-ischaemic injury. For example, there is diminished expression of the Tamm-Horsfall gene, and prepro-epidermal growth factor, as well as increased expression of immediate early genes c-fos and c-jun [Safirstein R 1994] and the calcium binding protein osteopontin [Padanilam BP 1996]. It is unclear what purpose these changes in gene transcription serve but it has been hypothesized they may have a paracrine function related to the close proximity of the thick ascending limb to the injured pars recta. Alternatively these genes may be involved in a non-proliferative pathway initiated by stress that is cytoprotective [Megyesi J 1995]. Calcyclin could be involved in such a pathway or its expression may represent an initial shift of cells into the G1 phase but with a subsequent halt in cell cycling and failure to progress to the S phase.

Further work is necessary to define calcyclin's role in ischaemic acute renal failure. It may provide a therapeutic target in the future as more knowledge becomes available concerning its function. The S100 protein family continues to be the source of intense research as involvement in more pathological conditions becomes apparent.
CHAPTER 4

INDUCED EXPRESSION OF CD27 AND ITS LIGAND SIVA IN RAT KIDNEYS FOLLOWING ISCHAEMIC INJURY

4.1 Introduction

It has traditionally been accepted that cellular necrosis is the main form of cell death following ischaemic renal injury. More recently experimental evidence has emerged that also supports an important role for apoptosis as a cause of cell death after renal ischaemia [Nakajima T 1996, Nogae S 1998, Raafat AM 1997, Yin T 1997]. The mechanisms involved in the initiation of apoptosis and its signalling pathways have been the focus of intense research. One of the possible mechanisms through which apoptosis may be initiated following ischaemia is through the activation of cell surface receptors. Other potential mechanisms through which apoptosis may be mediated are discussed earlier in Chapter 1.

The tumour necrosis factor (TNF) superfamily is the best known family of cell surface "death receptors" and includes Fas (also called CD95 or Apo1) and TNFR 1 (also called p55 or CD120a) [Nagata S 1995, Baker SJ 1998]. These receptors are known to play a very important role in cell growth and differentiation, as well as apoptosis. The homology is restricted to the extracellular region of the family members. TNFR 1 and Fas induced apoptosis is dependent upon the binding of their respective ligands, TNF-α and Fas ligand. Both receptors possess an intact 80 amino acid sequence termed the death domain in their cytoplasmic tails. Receptor ligation results in the clustering of the receptors' death domains and recruitment of adaptor proteins, which bind to the initiator procaspase-8 forming a death-inducing signalling complex. The caspases are a family of cell death
proteases that are crucial to the execution of apoptosis. So far at least 40 different protein substrates for the caspases have been identified including DNA repair enzymes [Nicholson DW 1997], nuclear structural proteins, cytoskeleton proteins and caspases themselves [Cohen GM 1997, Thornberry NA 1998].

There is some evidence that TNFR 1 and Fas induced apoptosis does occur following ischaemic renal injury. Renal tubule epithelial cells express both Fas [Boonstra JG 1997] and TNF-α [Jevnikar AM 1991] and addition of either anti-Fas antibodies [Boonstra JG 1997] or TNF-α to renal tubule epithelial cells induces apoptosis [Ostad M 1996]. Further evidence for a role of Fas has been provided by the demonstration of increased Fas and Fas ligand expression in the mouse model of ischaemic injury, and a reduction in the number of apoptotic cells in the lpr/lpr B6 mice which exhibit low levels of Fas expression as compared with the wild-type B6 mice following renal ischaemic injury [Nogae S 1998].

Recently a novel ligand for a member of the tumour necrosis factor receptor (TNFR) superfamily, CD27, has been cloned, and designated Siva [Prasad KVS 1997]. Siva has a death domain-like region that binds to the CD27 cytoplasmic tail in vitro. Over expression of Siva in a number of cell lines induces apoptosis, presumably through its association with CD27 in the cell membrane. Siva has been demonstrated in a number of non-lymphatic tissues such as prostate, testis, ovary and colon.

Using differential library screening we identified the induced expression of rat Siva following ischaemic renal injury. We further characterised its time course of expression, and its spatial expression in association with that of its receptor CD27 in ischaemic kidney. These findings represent the initial cloning of rat Siva and the first demonstrations of Siva and CD27 expression in the kidney. We propose that it is possible that an interaction
between the death domain of Siva and CD27 mediates apoptosis of renal cells following ischaemic injury.

4.2 Methods

Acute renal failure was induced by 60 minutes of bilateral renal artery clamping as described in Chapter 2 with sham-operated controls included at each point. To control for the extent of renal injury during the procedure, animals were selected such that the level of serum creatinine measured 24 hours following injury fell into the range used in previous studies. Levels of serum creatinine at 24 hours in groups of rats (mean ± SE, N = 3 rats) were 4.2± 0.3; 3.2± 0.5; 3.0± 0.3; and 3.5± 0.1 mg/dl in groups of rats used to obtain kidneys one, two, five, and seven days post-ischaemia, respectively (Table 4.1).

<table>
<thead>
<tr>
<th>No. of days post-ischaemia</th>
<th>Serum creatinine at 24 hours (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.2± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>3.2± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>3.0± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>3.5± 0.1</td>
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</tbody>
</table>

Table 4.1. Serum creatinine levels (mg/dl) at 24 hours in groups of rats used to obtain kidney 1, 2, 5 and 7 days following ischaemic renal injury.

Levels of serum creatinine were 1.0± 0.1 and 2.6± 0.1mg/dl (N = 3 rats) at one and 12 hours post-injury in the groups of rats that were sacrificed at these times (Table 4.2). In sham-operated rats serum creatinine levels were measured at the time of sacrifice and
averaged 0.6± 0.1 mg/dl (N = 36). These levels did not differ significantly from group to group.

<table>
<thead>
<tr>
<th>No. of hours post-ischaemia</th>
<th>Serum creatinine (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.0± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>2.6± 0.1</td>
</tr>
</tbody>
</table>

Table 4.2. Serum creatinine levels (mg/dl) in the groups of rats sacrificed at 1 and 12 hours post-ischaemic injury.

Levels of serum creatinine at the time of sacrifice of rats that had been rendered ischaemic were 2.0± 1.2, 0.8± 0.1, and 0.7± 0.1 mg/dl at two, five, and seven days post-injury, respectively (Table 4.3).

<table>
<thead>
<tr>
<th>No. of days post-ischaemia</th>
<th>Serum creatinine (mg/dl) at the time of sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.0± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>0.8± 0.1</td>
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<tr>
<td>7</td>
<td>0.7± 0.1</td>
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</table>

Table 4.3. Serum creatinine levels (mg/dl) at the time of sacrifice of rats that had been rendered ischaemic at 2,5 and 7 days.
4.2.1 Differential library screening

A small fraction of the acute renal failure cDNA library generated from kidneys of adult rats sacrificed 24 hours post-ischaemic injury was plated on a Nunc bioassay dish (Fisher, Pittsburg, PA, USA). Replica filters were made from the plates on zeta-probe membranes (BioRad, Hercules, California, USA). Total RNA was isolated from kidneys of sham-operated rats and rats rendered ischaemic at 24 hours post-surgery and was radiolabelled with [$^{32}$P]. One of the replica filters was then hybridised to cDNA probe from a sham-operated the kidney, and the second filter was hybridised to the probe from ischaemic kidney. The plaques that exhibited differential hybridisation were isolated and their differential expression confirmed by northern blot hybridisation.

4.2.2 DNA sequencing

Both strands of the cDNA were completely sequenced by primer walking on an automated AB1 Prism 377 sequencing machine. Sequences were searched against the GenBank nucleotide database using the BLASTN programme (Altschul SF 1990) and through a non-redundant protein database using the BLASTX programme.

4.2.3 Northern blot analysis

Northern blots were probed as described in Chapter 2 using a fragment containing bases -26 to 630 of the rat Siva cDNA (Figure 4.1). To confirm that equal quantities of RNA were loaded onto each lane, 18S and 28S bands on an agarose gel were stained using ethidium bromide, and it was determined that staining was the same in each lane. The band intensity after hybridisation was measured using a Phosphorimager and the statistical analysis performed as described previously. Comparison between gels was accomplished by normalising data to the value of the respective band originating from a kidney of a sham-operated rat.
Statistical analysis was performed using the student's t-test for unpaired sample means. P < 0.05 for 2-tailed analysis was considered significant.

4.2.4 In-situ hybridisation

In situ-hybridisation was performed on tissue sections originating from sham-operated rats and rats rendered ischaemic as described in Chapter 2. A rat Siva cDNA cloned in plasmid pBK-CMV (Stratagene, La Jolla, California, USA) was used as the template to generate digoxigenin-11-d UTP labelled sense and antisense ribo-probes. A fragment containing bases from - 26 to 630 was used as the sense and antisense probes.

4.2.5 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Deoxyribonuclease treated total RNA was reverse transcribed as described in Chapter 2 Twenty microlitres of the reaction was then PCR amplified using CD27 receptor specific oligonucleotides:

- \(5'\)-CACCTCCTACTGGCTCTG (forward primer)
- \(5'\)-CACTCTGTACATTCCTGGTC (reverse primer)

The oligonucleotides sequences were derived from the published mouse sequence (Gravestein LA 1993). The amplifying conditions were 40 cycles of the following: denaturation for one minute at 94°C, and annealing for 2 minutes at 56°C, and extension for one minute at 72°C. The PCR products were analysed in a 1% agarose gel stained with ethidium bromide, and the size of the product was estimated by comparison with a DNA ladder of known size. Agarose gel extraction of the PCR products was performed using the QIAEX II gel extraction kit (Qiagen, Valencia, CA, USA) The 348 base pair PCR fragment amplified from the rat RNA was cloned into the plasmid vector pGEM-T (Promega, Madison, Wisconsin, USA) and confirmed to represent the rat CD27,
homologue by sequencing characterisation. Control PCR amplifications were performed
in the absence of reverse transcriptase.

4.2.6 Western blotting

Western blotting was performed as described in Chapter 2 using M-20, a CD27-specific
primary antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA). M-20 is an
affinity-purified goat polyclonal antibody raised against a peptide corresponding to amino
acids 230 to 249 mapping at the carboxy terminus of the mouse CD27 precursor. M-20
reacts with CD27 of mouse and rat origin and is not cross reactive with other TNFR family
members.

4.2.7 Immunohistochemistry

Localisation of CD27 was performed by immunofluorescent staining of 5µm Bouins-fixed
paraffin-embedded sections using a modification of the method described in Chapter 2.
The primary antibodies were M20, obtained from Santa Cruz Biotechnology, Inc. The
sections were deparaffinized in xylene, rehydrated in graded ethanol, washed in phosphate-
buffered saline (PBS) followed by blocking in a PBS buffer containing 10% rabbit serum
and 0.2% Triton X-100 for 2 hour. The sections were incubated overnight at 4°C with the
primary antibodies (2µg/ml) in 1% rabbit serum and 0.2% Triton X-100. Cy3 linked
donkey anti-goat IgG was used as secondary antibodies. For blocking experiments, 2µg of
CD27 antibody was resuspended in 900µl of PBS and incubated overnight at 4°C with
10µg of blocking peptide (SC 1743p) or a scrambled peptide (SC 1744p) obtained from
Santa Cruz Biotechnology.
4.2.8 In-situ detection of apoptosis

Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end-labelling (TUNEL) staining was used to identify apoptotic cells.

4.3 Results

A cDNA library generated from kidneys from rats rendered ischaemic one-day previously was screened using cDNA probes generated from kidneys of either sham-operated rats or rats rendered ischaemic. Differential library screening identified several plaques that were: (1) either failed to hybridise or hybridised weekly to the probe originating from sham-operated rats; and (2) hybridised strongly to the probe from ischaemic rats. One clone, upon sequence characterisation was found to have high homology to the human proapoptotic protein, Siva cDNA.

Translation of the partial rat Siva open reading frame revealed a primary sequence 177 amino acids long (Figure 4.1A) with high homology (76 %) to the comparable portions of human Siva (Figure 4.1B), which is 189 amino acids long. Fifty-three of 75 amino acids are conserved within the death domain (71% homology; Figure 4.1B). Therefore, the homology between the death domains of rat and human Siva is higher than that between human Siva and the death domains of the human proteins FADD or RIP (40%). Twenty-nine of the 32 amino acids in the B-box-like ring finger region of rat Siva are homologous to those in human Siva (Figure 4.1B).
Figure 4.1 (A) cDNA and deduced amino acid sequence for rat Siva, and (B) comparison between rat (R) Siva and human (H) Siva. D defines the death domain and B defines the B-box-like region.
To characterise the time course of renal rat Siva expression post-ischaemic injury, kidneys were obtained from sham-operated rats and rats rendered ischaemic, one and 12 hours, and one, two, five, and seven days post-sham surgery or post-ischaemia, and Northern assays were performed using RNA extracted from the kidneys. A representative autoradiogram of a gel from a Northern assay originating from mRNA extracted from three kidneys obtained five days post-ischaemia or sham surgery is shown in Figure 4.2 (upper right). Rat Siva mRNA (800 base pair band) was visibly increased in kidneys from rats rendered ischaemic relative to levels in kidneys from sham-operated animals.

Levels of rat Siva mRNA in kidneys of three groups of rats subjected to sham-surgery or ischaemic injury at 12 hours, and one, two, five or seven days prior to sacrifice were determined by Northern analysis were quantified using a Phosphorimager. The level of rat Siva mRNA in kidneys from rats subjected to ischaemic injury was not significantly increased compared to that in kidneys from sham-operated rats at one-hour post-injury. However, by 12 hours, rat Siva mRNA levels were increased by approximately 4.5-fold. Levels of rat Siva mRNA in kidneys from rats subjected to injury were elevated significantly (P < 0.05) compared to levels in kidneys from sham-operated rats at 12 hours, and one, five and seven days post-injury, but not at two days post-injury (Figure 4.2).
Figure 4.2 Northern assay for Siva mRNA and the effect of ischaemia on renal Siva mRNA levels. (Inset) A representative autoradiogram of a Northern blots generated using RNA from kidneys of three rats that underwent sham-surgery and three rats rendered ischaemic 5 days previously. The size of the RNA species (800 base pairs) is shown. Also shown are results from three Northern assays quantified using a phosphorimager. N = 3 rats in each group. Data are mean ± SE. ARF > sham at 12 hours, and at 1, 5 and 7 days (P < 0.05, Student's t-test). Symbols are (□) sham; (■) ARF.
In-situ hybridisation demonstrated that rat Siva mRNA expression in kidney did not differ among sham-operated rats sacrificed at varying times post-surgery (not shown). Figures 4.3 and 4.4 show photomicrograph sections originating from the inner cortex and outer medulla of kidneys in sham-operated rats (Figure 4.3D) or ischaemic rats (Figure 4.3 A-C, E, F) sacrificed at 12 hours (Figure 4.3 A-C), one day (Figure 4.4 A-C), five days (Figure 4.3 D-F), or seven days (Figure 4.4 D,E) post surgery. Little or no hybridisation is observed when a sense control probe is used at 12 hours (not shown) or five days post-ischaemia (Figure 4.3 F). Little rat Siva mRNA is present in tubules of kidneys from sham-operated rats (Figure 4.3 D). Rat Siva mRNA is localised to cells within ischaemic tubules of the S3 proximal segment at 12 hours post-injury (Figures 4.3 A-C), and at one-day post injury (Figure 4.4 A) and in papillary proliferations (p) within regenerating tubules at five days post-injury (Figure 4.3 E), and seven days post-injury (Figure 4.4 D). In addition to papillary proliferations, rat Siva mRNA is expressed in simplified (flattened) epithelial cells lining dilated regenerating segments (Figure 4.3 E). Siva mRNA is expressed at no other locations in the kidney. A sense probe was used in figure 4.3 F, which demonstrated no signal.

In non-serial sections, TUNEL positive cells were present at the same locations as were cells staining for Siva mRNA that had been sloughed into ischaemic tubules in the S3 segment, or that were present in papillary proliferations. This is illustrated in photomicrographs of kidneys obtained from rats one-day post-ischaemia (Figure 4.4 A, C) or seven days post-ischaemia (Figure 4.4 D, E). Figure 4B shows the results of a control experiment performed without TdT.
Figure 4.3 Localisation of renal Siva mRNA by non-isotopic in-situ hybridisation. Siva mRNA in kidneys from rats are subjected to sham-surgery (D) or rendered ischaemic (A-C,E,F) 12 hours (A-C) or 5 days prior to sacrifice (E,F), was localised using a digoxigenin labelled antisense riboprobe (A-E) or sense probe (F). Arrow points towards renal papilla (B). Arrowhead shows ischaemic proximal tubule (C). P, papillary proliferation (E,F). Sections are representative of > 5 experiments. A to C are progressive enlargements of the same section. Magnification (15 μm) is shown for C-F (F).
Figure 4.4 TUNEL staining and localisation of renal Siva mRNA by non-isotopic in-situ hybridisation. TUNEL staining was performed in kidneys from rats rendered ischaemic 1 day (B,C) or 7 days (E) prior to sacrifice. TdT was omitted in the experiment illustrated in B. Siva mRNA in kidneys from rats rendered ischaemic 1 day (A) or 7 days prior to sacrifice (D) was localised as in Figure 4.3. Arrowheads show cells sloughed within ischaemic renal tubules (A-C). P, papillary proliferation (D,E). Non-serial sections are shown representative of > 5 experiments. Magnification (10μm) is shown (E).
To determine whether CD27, the ligand for Siva, is expressed in rat kidney, we amplified cDNA in reverse-transcribed extracts of mouse or rats kidneys using PCR and CD27-specific primers, and performed western blots of rats renal extracts. As shown in figure 4.5 A, CD27 mRNA was present in mouse (M) and rat (R) kidney extracts. The size of the amplified cDNA product (348 base pairs) was identical. No band was observed if reverse transcriptase was omitted from the reaction (C). Western blots of proteins extracted from rat kidneys showed antibody binding to a single 55kD band in membrane (Mm), but not in soluble (S) fractions of rat kidney (Figure 4.5 B). These results show that CD27 is present in kidney as a transmembrane dimer composed of 55kD monomers, as expected [Kobata T 1994].
Figure 4.5 (A) Reverse transcribed-polymerase chain reaction (RT-PCR) of rat kidney RNA extract and (B) Western blot of rat kidney extracts. (A) Size markers (left) and the size of the amplified CD27 cDNA product is shown (right). Abbreviations are: C, control (no reverse transcriptase); M, mouse kidney; R, rat kidney. (B) The size of the CD27 monomer is shown (right). Abbreviations are: Mm, membrane fraction; S, soluble extract.
The localisation of CD27 protein in kidney is shown in figure 4.6. In sections originating in kidneys obtained 12 hours (Figure 4.6 B) and five days (Figure 4.6 C) post-ischaemia, immunoreactivity is observed only in sloughed cells within the S3 segments of the proximal tubule (Figure 4.6 B) and in papillary proliferations (p) (Figure 4.6 C). Unlike rat Siva mRNA (Figure 4.3 C), CD27 protein is absent from epithelial cells lining regenerating tubules (Figure 4.6 C). Figure 4.6 A, D, and E show tissue for which the control antibody was substituted for anti-CD27 (Figure 4.6 A), or tissue exposed to anti-CD27 together with CD27 scrambled peptide (Figure 4.6 E), or blocking peptide (Figure 4.6 D). Staining was inhibited by blocking peptide (Figure 4.6 D), but not scrambled peptide (Figure 4.6 E).
Figure 4.6 Immunohistochemical localisation of CD27 in kidneys. CD27 peptide in kidneys from rats rendered ischaemic 12 hours (A,B) 24 hours (D,E) or 5 days (C) prior to sacrifice was localised using an antibody generated against CD27 (B,C), anti-CD27 together with blocking peptide (D), scrambled peptide (E) or control antibody (A). Arrowhead points out sloughed cells within the lumen of tubules (B). Papillary proliferations are shown (p) (C). Sections are representative of > five experiments. Magnification (10 μm) is shown (C).
4.4 Discussion

Apoptosis occurs in an orchestrated fashion during kidney development. In contrast it is a relatively rare event in normal developed (adult) kidney. However, following induction of acute ischaemic renal injury in the rat, apoptosis is observed in regenerating tubules [Schumer M 1992, Shimizu A 1993], during which, parts of the developmental pattern are recapitulated. Apoptotic cells at the site of regeneration following ischaemic injury in the rat kidney have been identified by light microscopy, electron microscopy and, to our knowledge for the first time using TUNEL-staining (Figure 4.4).

Previous work has demonstrated that following the induction of acute ischaemic renal injury in rats two peaks of apoptosis occur in regenerating tubules[Shimizu A 1993]. The first peak coincides with a burst of proliferative activity that occurs at two days post-injury. Between days three and six post-ischaemia, the percentage of cells undergoing apoptosis falls. However, the incidence of apoptosis increases again on day 7 and is maximal on day 8 following injury, after which the incidence again declines slowly over a period of weeks. Hyperproliferation during days 2 to 3 results in a stratification of hyperplastic epithelial cells that is demonstrable two to four days post injury and in a hyperplasia of circumferential epithelial cells with focal papillary projections that are evident at five to seven days [Basile DP 1996]. The hyperplastic tubules are returned to their original cellularity and papillary proliferations are remodelled through a wave of apoptosis that is reflected by the second peak. The existence of a predictable pattern of apoptosis in kidney during recovery following ischaemic injury indicates that this process is an important one.

Apoptotic cell death is frequently observed in human ATN biopsy specimens and in animal models following ischaemic renal injury. There are multiple factors that are known to induce tubular cell apoptosis and these factors can be divided into several categories. First,
a lack of survival signals from a relative deficiency in soluble growth factors and from loss of normal cell-cell and cell-matrix interactions; second, cytotoxic stimuli such as increased calcium, reactive oxygen species and numerous nephrotoxic drugs; and third, various receptor-mediated mechanisms. Recent work in a rat model of ischaemic acute renal failure has confirmed our findings of cell death occurring by apoptosis mainly in the outer medulla peaking at 24 hours [Jo SK 2001]. Most apoptotic cells were detached from the tubular basement membrane and found in the tubular lumen. In this study the investigators demonstrated an increase expression of Fas and Fas ligand occurring at 72 hours following ischaemia. It was therefore unlikely that the Fas and Fas ligand expression was responsible for the initial wave of apoptosis. In this study they did not extend the period of observation following ischaemic injury beyond 72 hours.

Other attempts have been made to characterise the expression of factors known to regulate programmed cell death. The bcl-2 family of proteins is the best characterised family of apoptotic effectors. It has been previously shown that the expression of the regulatory proto-oncogenes, bcl-2 and bax, are enhanced in regenerating S3 segments following ischaemic injury in the rat [Basile DP 1997]. The changes in bcl-2 and bax expression that we have previously described post-ischaemia may or may not control apoptosis in regenerating S3 segments. However, bcl-2 is known to play a major anti-apoptotic role during kidney development [Sorenson CM 1995]. Since the process of renal regeneration post-ischaemia is similar in many ways to development, a role for bcl-2, and perhaps for the regulated protein bax, in the former process would not be unexpected.

Enhanced expression of members of the TNFR superfamily and ligands for these receptors occurs in ischaemic kidney [Kato S 1997, Ortiz-Arduan A 1996] and in ischaemic organs in addition to kidney. For example, hepatic ischaemic/reperfusion injury in rats results in
tumour necrosis factor (TNF) production in liver. Pretreatment of rats rendered ischaemic with anti-TNF antiserum attenuates the injury [Colletti LM 1990].

It has been proposed that the relative overproduction of TNF amplifies and extends the severity of cell death during the brain ischaemia. Therapeutic implications for this proposal are demonstrated by the attenuation of brain damage post-ischaemia following in the intra-cerebroventricular administration of anti-TNF antibodies or receptor constructs [Botchkina GI 1997].

CD27 is a member of the tumour necrosis factor receptor (TNFR) superfamily and is known to be expressed in T and B lymphocytes. The homology is restricted to the extracellular region of the family members and is characterised by the presence of a Cys knot motif, which occurs three times in CD27. CD27 is a glycosylated, type 1 transmembrane protein of about 55kDa and exist as homodimers with a disulphide bridge linking the two monomers. Expression of CD27 has previously been demonstrated on discrete populations of both T and B cells and as well as being detected in the serum of normal individuals. CD70 is the ligand for CD27, and belongs to the TNF family of ligands. It is a type II transmembrane protein with an apparent molecular mass of 50 kDa. Unlike TNF there are no reports as to the existence of a naturally occurring soluble form of CD70. The interaction of CD27 with its ligand CD70, also expressed in T and B cells, can provide costimulatory signals for T and B cell proliferation and for immunoglobulin production in B cells [Prasad KVS 1997, Hintzen RQ 1994]. Unlike TNFR I and Fas, the cytoplasmic tail of CD27 lacks a death domain. However, the interaction between CD70 and CD27 can induce apoptosis.
We have demonstrated that a rat homologue of the human gene Siva is expressed in the S3 segments of proximal tubules and in papillary proliferations at times (12 hours to 1 day and 5 to 7 days post-injury, respectively) that roughly parallel the two peaks of apoptosis that occur at this site post-ischaemia. In contrast, enhanced expression is not observed at an intermediate time (2 days post-injury). CD27, a ligand for Siva, is expressed in injured kidney tissue at 12 hours and 5 days post-injury within sloughed cells (12 hours) and cells within papillary proliferations (5 days; Figure 4.6). In each of these locations at each of these times, its ligand, Siva, is also expressed (Figure 4.3). These are the cell populations known to be undergoing death via apoptosis or necrosis at these times (Figure 4.4). Our findings are consistent with a role of Siva acting through CD27 in the apoptosis that occurs in the rat proximal tubule following ischaemic injury.

Evidence is now starting to accumulate that the inhibition of apoptosis following ischaemia attenuates the degree of renal injury in the rat model. The administration of α-melanocyte stimulating hormone (α-MSH) significantly reduced the degree of apoptosis following ischaemic acute renal failure as measured by serum creatinine and by the degree of histological damage [Jo SK 2001]. The suggested mechanisms of α-MSH in decreasing apoptosis has been suggested to include an inhibitory effect on neutrophil infiltration and inflammatory cytokine production. Further work using a murine model of ischaemic renal injury has demonstrated that the administration of ZVAD-fmk (a caspase inactivator) prevents the early onset of renal apoptosis with a reduction in renal injury [Daemen MARC 1999].

Our own work is the first demonstration of Siva and CD27 expression in the kidney, and proposes a novel pro-apoptotic ligand/receptor interaction following ischaemic renal injury. The ligation of CD27 with Siva can induce apoptosis in-vitro, but the mechanism
by which CD27 ligation with Siva induces apoptosis in-vitro is unknown. Furthermore, the existence of CD27-Siva protein complexes in-vivo and the role of Siva in CD27-mediated apoptosis in vivo are unproven. Our demonstration that Siva and CD27 are expressed in kidney following renal ischaemia does not define a causative role of Siva-CD27 for renal apoptosis. Nonetheless, as is the case in brain for TNF and TNFR, it is possible that strategies directed at modifying CD27-mediated renal apoptosis will impact positively on the course of acute ischaemic renal injury. Their success will depend on an expanded understanding of the role that apoptosis plays in the regenerative process, as they could be aimed at accelerating the programmed death of damaged cells [Hammerman MR 1998, Savill J 1994] or at limiting it [Lieberthal W 1996].
CHAPTER 5

EXPRESSION OF CD44 AND ITS LIGANDS IN RAT KIDNEY FOLLOWING ACUTE ISCHAEMIC INJURY

5.1 Introduction

The CD44 family of cell surface glycoprotein receptors is widely expressed in embryonic, normal adult, and neoplastic tissues. CD44s serve as adhesion molecules in cell:cell and cell:substrate interactions that mediate processes such as cell migration during organogenesis or wound repair, and metastasis [Borland G 1998, Naor D 1997]. Hyaluronic acid, an ubiquitous polysaccharide synthesized by fibroblasts, chondrocytes and mesothelial cells is the major ligand for CD44 [Borland G 1998, Naor D 1997, Sibalic V 1997]. However, CD44 can bind other ligands including osteopontin [Singh K 1992, Weber GF 1996], a secreted phosphoprotein expressed at many epithelial cell surfaces in communication with the outside environment including the nephron [Padanilam BJ 1996, Rodan GA 1995].

Little is known about the role of CD44 in the kidney. During embryogenesis transient interference of CD44 expression by intravenous injection of antibody into pregnant rats results in the delay in the development of the tubular system of the kidney [Zoller M 1997], thereby implying a role in renal development. In kidneys of normal rats, CD44 has been reported to be undetectable [Wirth K 1993] or detectable in intrinsic glomerular cells, parietal epithelial cells of Bowman's capsule, medullary tubules, and occasional cortical tubules (thick ascending limb of Henle's loop and distal tubules) [Jun Z 1997, Nikolic-Paterson DJ 1996].
Under normal conditions in humans, CD44 is undetectable [Terpe HJ 1994], or barely detectable in the distal tubule [Mackay CR 1994, Terpe HJ 1994] by immunohistochemistry. However, consistent with its role as a mediator of cancer cell proliferation and migration, CD44 is easily detected in the cell membranes of malignant, but not benign renal cell tumors in humans [Terpe HJ 1996].

The expression of CD44 has been described in a variety of other pathological conditions. De-novo CD44 and ligand expression occurs at wound margins and accompanies cellular proliferation and migration that effect repair of injured vascular endothelial [Jain M 1996] and epithelial [Nikolic-Paterson DJ 1996] tissues. It has also been suggested that the interaction of CD44 with its ligands, osteopontin and hyaluronic acid could participate in the tubulointerstitial inflammatory response seen in the kidneys of *kdkd* mice. These mice develop a progressive cell-mediated tubulointerstitial nephritis [Sibalic V 1997]. CD44 is co-expressed de-novo with osteopontin in injured proximal tubule epithelial cells of *kdkd* mice, whilst hyaluronic acid accumulates in the interstitial space around these injured tubules.

Hyaluronic acid is a high molecular-mass polysaccharide found in the extracellular matrix, especially of soft connective tissues [Laurent TC, 1992]. It is synthesised in the plasma membrane of fibroblasts and other cells by the addition of sugars to the reducing end of the polymer, whereas the non reducing end protrudes into the pericellular space. Hyaluronic acid production increases in proliferating cells [Laurent TC, 1988] and the polymer may play a role in mitosis [Brecht M 1986]. A possible function would be that hyaluronic acid, growing out from the cell surface, causes a detachment of the cell from supporting matrix so that it can divide more easily. In vitro cell detachment mediated by hyaluronic acid has been described. Hyaluronic acid always seems to surround proliferating and migrating
cells in regenerating, remodelling or healing tissues [Toole BP 1984]. Indeed, hyaluronic acid has been used to promote wound healing. Application of hyaluronic acid in the middle ear of rats [Hellstrom S 1987] results in more rapid healing of perforated tympanic membranes. It plays an important role during development and differentiation. Hyaluronic acid concentration often increases in compartments were cellular migration is going to take place. It has been suggested that the polysaccharide actually promotes locomotion [Turley EA 1989].

Hyaluronic acid can be measured in the blood and increased serum levels can be seen in a variety of inflammatory conditions such as rheumatoid arthritis, psoriasis and scleroderma. Excess hyaluronic acid can promote interstitial oedema and organ dysfunction. It's accumulation has been demonstrated in renal transplants undergoing acute rejection [Wells AF 1990].

Osteopontin is a highly acidic phosphoprotein that was first isolated from bone but is also produced in other tissues including different epithelial cells of the kidney, respiratory, gastrointestinal tract, frequently in association with their luminal surface. The protein is secreted and binds in the extracellular fluid to fibronectin and collagen [Beninati S 1994]. Osteopontin contains an arginine-glycine-aspartic acid (RGD) sequence that is highly conserved among diverse species. It is therefore able to bind to integrin receptors promoting cell adhesion, spreading and locomotion. Osteopontin is also able to bind non-integrin receptors such as CD44. Despite the significant progress in understanding the structure of osteopontin its functional role remains insufficiently understood. It has been demonstrated to be elevated in some cancers, under the conditions of sepsis and inflammation and may participate in wound healing [Rodan GA 1995], which has led to the suggestion that osteopontin is important for tissue repair and remodelling. An anti-
inflammatory role has been implied by the demonstration of osteopontin-induced suppression of the inducible nitric oxide synthase (iNOS) [Hwang S 1994].

Recovery of renal function following ischaemic acute renal failure is dependent upon a process of re-epithelialisation affected via cellular proliferation and migration across portions of the denuded proximal tubule basement membrane. To determine whether de-novo expression of CD44 and its ligands might play a role in the process of recovery post-ischaemic injury in the regenerating proximal tubule, we characterized the expression and localization of CD44 and two of its ligands, hyaluronic acid and osteopontin, in kidneys of rats that underwent renal ischemia and in kidneys of sham-operated controls. Our data are consistent with CD44-ligand interactions playing a role in the process of proximal tubule regeneration after renal ischaemia.

5.2 Methods

Acute renal failure was induced by 60 minutes of bilateral renal artery clamping as described previously in Chapter 2. To control for the extent of renal injury during the procedure, animals were selected such that the level of serum creatinine measured 24 hours following injury fell into the range, used in previous studies [Basile DP 1996, Padanilam BJ 1996]. Levels of serum creatinine measured 24 hours post-ischaemia (mean ± SE) were 4.2 ± 0.3, 3.2 ± 0.5, 3.2 ± 0.5, 3.0 ± 0.3, and 3.5 ± 0.1 mg/dl in groups of rats used to obtain kidneys 1, 2, 3, 5, and 7 days post-ischaemia respectively (Table 5.1).
Table 5.1 Serum creatinine levels (mg/dl) at 24 hours in groups of rats used to obtain kidney 1,2,3,5 and 7 days following ischaemic renal injury.

Levels were 2.6 ± 0.1 mg/dl (n = 3 rats), 12 hours post-injury in the group of rats that was sacrificed at this time. Levels of serum creatinine in sham-operated rats were measured at the time of sacrifice and averaged 0.6 ± 0.01 mg/dl (n=27). These levels did not differ significantly from group to group.

Table 5.2 Serum creatinine levels (mg/dl) at the time of sacrifice of rats that had been rendered ischaemic at 2,3,5 and 7 days.
Levels of serum creatinine at the time of sacrifice of rats that had been rendered ischaemic were 2.0 ± 1.2, 1.9 ± 0.9, 0.8 ± 0.1, and 0.7 ± 0.1 mg/dl at 2, 3, 5 and 7 days post-injury respectively (Table 5.2).

5.2.1 Northern blot analysis

A specific cDNA probe for CD44 was generated from a 1228 base-pair clone that was kindly provided by Dr. M. Jain (Beth Israel Hospital, Harvard Medical School, Boston MA) [Jain M 1996].

Using the polymerase chain reaction (PCR), T3 and T7 RNA polymerase promoter sequences were added to an amplified CD44 cDNA. The specific T3 and T7- linked oligonucleotides were:

- 5' AATTAACCCTCACTAAAGGGTATATCCTCCTCGCATCCA 3'
- 5' TAATACGACTCACTATAGGGTCCTGTCTTCCACTGTTCC 3'.

The synthesis of a 667 base pair PCR product was confirmed using ethidium bromide-agarose gel electrophoresis. Restriction enzyme digest demonstrated cDNA fragments of the predicted size for CD44. The CD44 PCR product was radiolabelled with [32P-dCTP] (Amersham, Arlington Heights IL) using a random prime labelling kit (Stratagene, LaJolla, CA).

In order to determine that equivalent amounts of RNA were present in each lane, an ethidium bromide-agarose gel containing aliquots of the same RNA used for Northern blots was photographed under ultraviolet light as before [Basile DP 1996].
5.2.2 In-situ hybridisation

The 667 base pair CD44 cDNA fragment was used as the template to generate sense and antisense probes for in-situ hybridisation.

5.2.3 Immunohistochemistry

Immunohistochemistry was performed as described previously in Chapter 2. The primary osteopontin antibody MOIIIB10 was obtained from the developmental studies hybridoma bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, and the department of Biological Sciences, University of Iowa, Iowa City, IA. The bank is maintained under contract NO1-HD-6-2915 from the National Institute of Child Health and Human Development. The osteopontin antibody was applied in the 1/10th concentration of the blocking buffer overnight at 4 °C. The primary CD44 antibody was mouse anti-rat monoclonal antibody OX49 (Pharminen, San Diego CA), and was used at a concentration of 1:50. As controls, non-immune cellular supernatant obtained from the same source as MOIIIB10 was substituted for MOIIIB10 and mouse IgG1 was substituted for OX49. Positive staining was not observed in control experiments.

The biotinylated proteoglycan (b-PG) fragments used to localize hyaluronic acid were kindly provided by Dr. C. Underhill (Georgetown University, Washington DC). Tissue sections were incubated with 8 µg/ml of b-PG in 10% goat serum and 90% PBS overnight at 4 °C. Immunohistochemistry was performed as described above. As a control, tissue sections were incubated in b-PG that has been absorbed with 0.1 mg/ml hyaluronic acid.
5.3 Results

To determine whether CD44 is expressed in rat kidney following ischaemic injury, we first performed a Northern analysis of RNA extracted from kidneys of sham-operated rats (S) or rats with acute renal injury (A) rendered ischaemic 1, 3, 5 or 7 days prior to sacrifice (Figure 5.1). A heterogeneous mixture of mRNAs was present in kidneys from rats rendered ischaemic, but not in kidneys from sham-operated rats. The sizes of transcripts (4.5 kb, 3.3 kb, 2.0 kb and 1.6 kb) were identical to those reported elsewhere (Jain M) in rat vascular smooth muscle cells. Multiple transcripts could have been generated by alternative splicing or by polyadenylation.
Figure 5.1 Northern blot of CD44 mRNA. Total RNA was isolated from kidneys originating from sham operated rats (S) or rats with acute renal injury (A) rendered ischaemic 1, 3, 5 or 7 days prior to extraction. Northern blotting was performed using a radiolabelled CD44 probe. Four transcripts are visible at 4.5, 3.3, 2.0 and 1.6 kb. An ethidium bromide-stained gel with 28S and 18S bands demarcated is shown to verify equivalent loading.
CD44 mRNA localisation in kidney post-ischaemia was demonstrated by in-situ hybridisation using a CD44-specific antisense probe or a sense probe (Figure 5.2). Little or no CD44 mRNA is expressed in kidneys from sham-operated rats (Figure 5.2 A). No staining is observed when sense riboprobe is substituted for the antisense probe (Figure 5.2 B). However CD44 mRNA is expressed in regenerating S3 proximal tubules in the inner cortex/outer medulla by 1 day post-injury (Figure 5.2 C), which can be seen more clearly at high magnification (Figure 5.2 D) and continues to be expressed on days 3 (Figure 5.2 E), 5 (Figure 5.2 F) and 7 post-ischaemia (Figure 5.2 G).
Figure 5.2 Localisation of renal CD44 mRNA by non-isotopic in-situ hybridisation. CD44 mRNA in kidneys from rats subjected to sham-surgery (S) 1 day prior to sacrifice or rendered ischaemic 1-7 days prior to sacrifice (1-7D ARF) was localised using a digoxigenin labelled antisense riboprobe or a sense riboprobe where indicated: A) 1D S; B) 1D ARF, sense riboprobe; C) 1D ARF; D) 1D ARF; E) 3D ARF; F) 5D ARF; G) 7D ARF, anti-sense probe. oc, outer cortex, ic, inner cortex (C). Arrowheads show proximal tubules (D-G); Sections are representative of > 5 experiments. Magnification is shown for A.
Immunohistochemistry confirmed that the changes in CD44 mRNA were accompanied by changes in the levels of CD44 peptide. There is little or no CD44 staining in kidneys originating from sham-operated rats (Figure 5.3 A) or in rats rendered ischaemic 12 hours post-injury (Figure 5.3 B). However, CD44 is expressed in proximal tubules by 1 day post-injury (Figure 5.3 C and E) and at 5 days post-injury (Figure 5.3 D and F). Enlargement of Figure 5.3 C shows that CD44 is expressed along the basal and lateral membranes of regenerating tubules (Figure 5.3 E) (arrowheads).

The data shown in Figures 5.1-5.3 establish that CD44 is up-regulated in regenerating proximal tubules following ischaemic injury. To shed light on the identities of possible ligands for CD44 in this setting, we localized hyaluronic acid and osteopontin, in sections of kidneys obtained from rats rendered ischaemic and from sham-operated controls.
Figure 5.3 Immunohistochemical localisation of CD44 peptide in kidneys. CD44 peptide in kidneys from rats subjected to sham-surgery (S) or rendered ischaemic (ARF) 12 hours (12h), 1 day (1D) or 5 days (5D) prior to sacrifice was localised using an antibody generated against CD44: A) 12h S; B) 12h ARF; C) 1D ARF; D) 5D ARF; E) 1D ARF; F) 5D ARF. Arrowheads show basal and lateral membranes of proximal tubule cells (E). Sections are representative of > 5 experiments. Magnification is shown for A-D and E,F.
It has been reported previously that hyaluronic acid is localized in rat kidney to the renal papilla in which it surrounds the interstitial cells [Girard N 1986]. The use of b-PG shows that hyaluronic acid is expressed in the renal papilla of kidney from sham-operated controls (Figures 5.4 B) as previously reported [Girard N 1986].

In kidney sections obtained 1 day post-sham surgery, incubation with absorbed b-PG results in negative staining (Figure 5.4 A). No hyaluronic acid staining is observed in the cortex of kidneys from sham-operated rats (Figure 5.4 D). By 1 day post-ischaemia (Figure 5.4 E) and at 3 days (Figure 5.4 F) and 5 days (Figure 5.4 G) post-ischaemia, hyaluronic acid is present in the renal cortex and is localised in the interstitium between regenerating proximal tubules (Figure 5.4 E-G), but not in the tubules themselves. The margins of the proximal tubules cells can be delineated in Figure 5.4, E-G. There is still a small degree of hyaluronic acid staining evident in the interstitium by 28 days post-ischaemic injury (Figure 5.4 H).
Figure 5.4 Localisation of hyaluronic acid in kidneys. Hyaluronic acid peptide in kidneys from rats subjected to sham-surgery (S) or rendered ischaemic 1-28 days prior to sacrifice (1-28D ARF) was localised using b-PG or a control (absorbed b-PG) when indicated: A) 1D S control; B) 1D S; C) 1D S; D) 1D S; E) 1D ARF; F) 3D ARF; G) 5D ARF; H) 28D ARF. Arrows show cortico-medullary junctions (A,B); p delineates direction of papilla (B); g, glomerulus (D); arrowheads delineate margins of proximal tubule epithelial cells (F,G). Sections are representative of > 5 experiments. Magnification is shown for E and H (E).
We have shown previously that the expression of osteopontin in the distal tubule and medullary thick ascending limb (MTAL) of Henle's loop is enhanced within 1 day of inducing acute ischaemic renal injury in the rat [Padanilam BJ 1996]. In addition, the co-expression of osteopontin and osteopontin mRNA, is observed in regenerating proximal tubules at 5 days following ischaemia [Padanilam BJ 1996]. To better delineate the time-course of enhanced osteopontin expression in the regenerating proximal tubules, we performed immunohistochemistry for osteopontin in kidneys obtained from rats, 12 hours, 1, 3, 5 and 7 days following injury and following sham surgery (Figure 5.5).

As in kidneys from normal rats, the use of control antibody resulted in no staining (Figure 5.5 A) and no staining for osteopontin was observed in proximal tubules in kidneys obtained from sham-operated rats (Figure 5.5 B). Enhanced staining in distal tubules (d), but not in damaged proximal tubules (arrowheads) was observed beginning as soon as 12 hours following injury (Figure 5.5 C) and at 1 day (Figure 5.5 D). By 3 days post-ischaemia, cells lining regenerating tubules stained for osteopontin (Figure 5.5 E), as was the case at 5 days (Figure 5.5 F).
Figure 5.5 Immunohistochemical localisation of osteopontin peptide in kidneys. Osteopontin peptide in kidneys from rats subjected to sham-surgery (S) or rendered ischaemic (ARF) 12 hours - 7 days (12h-7D) prior to sacrifice was localised using an antibody generated against osteopontin or, when indicated, a control antibody: A) 1D ARF stained with control antibody; B) 1D S; C) 12h ARF; D) 1D ARF; E) 3D ARF; F) 5D ARF; G) 7D ARF. Arrowheads show proximal tubules (C-G). Sections are representative of > 5 experiments.
Figure 5.6 shows serial sections of renal cortex originating from rats rendered ischaemic 3 days (A and B) or 7 days (C and D) prior to sacrifice. The identical regenerating tubules are represented in Figure 5.6 A and B or Figure 5.6 C and D. Co-expression of osteopontin (Figure 5.6, A and C) and CD44 (Figure 5.6, B and D) can be demonstrated in some (+) regenerating tubules. Osteopontin and CD44 are present in some of the same cells (arrowheads). As previously reported [Padanilam BJ 1996] osteopontin is expressed in distal tubules (d) of regenerating kidneys (Figure 5.6 C). However, CD44 is not co-expressed at this site (Figure 5.6 D).
Figure 5.6 Immunohistochemistry of serial sections for osteopontin and CD44. Serial sections of rat kidneys obtained from a rat rendered ischaemic 3 days (A,B) or 7 days (C,D) prior to sacrifice stained for: osteopontin (A,C); or CD44 (B,D). (+) delineates a regenerating tubule that stains for both peptides. Arrowheads show cells that stain for both peptides. d, distal tubule (C,D). Sections are representative of 5 experiments.
5.4 Discussion

CD44:ligand interactions are thought to play important roles in tissue development and repair through the mediation of cell binding to endothelium or to the extracellular matrix [Naor D 1997]. One source of evidence for such involvement in these processes is the localisation of CD44 at sites of active cell proliferation and migration. In 10 week-old human embryos, CD44 is found in dividing cells of the epidermis, trachea, lung, thyroid gland and mesonephric ducts [Terpe HJ 1994]. In newborn rats CD44 has been identified in basal layers of the epidermis, hair follicles, the lower parts of crypts in colon mucosa and ductal epithelia of pancreatic glands [Wirth K 1993].

CD44 is normally present in cell membranes of all layers of the stratifying epithelium in the developed human palate except for the stratum corneum. Within 1 day following experimental full-thickness wounding of the palate mucosa, a fibrin clot occupies the wound space, and at the margins, sheets of epithelial cells begin to migrate into the wound. The first migrating epithelial cells are weakly CD44 positive. However, by 3 days post-injury, they are markedly positive. By day 1, the lateral sides of the wound bed stain positive for the CD44 ligand, hyaluronic acid. CD44 in the plasma membrane of migrating cells, and hyaluronic acid in the wound margin and connective tissue matrix, are co-expressed during all tissue repair stages, suggesting that the CD44:matrix-hyaluronic acid interaction plays an important role in re-epithelialisation [Nikolic-Paterson DJ 1996].

Similar to events in the palate mucosa, CD44 mRNAs are up-regulated in rat carotid arteries after balloon-induced injury. CD44 protein expression is greatest at the luminal edge of the growing neointima. CD44-expressing smooth muscle cells proliferate actively after injury and hyaluronic acid expression increases at the same time throughout the neointima [Jain M 1996].
Osteopontin is normally expressed at low levels in the renal distal tubule and medullary thick ascending limb of Henle's loop [Padanilam BJ 1996]. Within 1 day following acute ischemic injury in the rat, its expression is enhanced at these sites [Kleinman JG 1995, Padanilam BJ 1996]. Later, during the process of regeneration, osteopontin is expressed in regenerating proximal tubules [Padanilam BJ 1996]. It should be noted that the staining pattern of osteopontin is different dependent upon its sites of expression. In the regenerating proximal tubules the osteopontin staining is vesicular and perinuclear, whereas in the distal tubules osteopontin is present at the apical cell side. The importance of osteopontin expression in the process of recovery is underscored by the observation that transgenic mice unable to express osteopontin do not recover normally from acute ischaemic injury [Noiri E 1999].

Ligands for osteopontin in addition to CD44, include integrins, primarily $\alpha_v\beta_3$, to which it binds via an arginine-glycine-aspartate (RGD)-motif [Rodan GA 1995]. The integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$ also serve as osteopontin receptors on vascular smooth muscle cells [Rodan GA 1995]. In the kidney, binding sites for RGD have been detected along the basolateral membrane of proximal tubule cells. Within 1 day following ischaemic injury, sites are increased along the basolateral membrane and can be detected also along the apical membrane, in peritubular capillaries and on desquamated cells within tubular lumens [Romanov V 1997]. RGD binding-sites within regenerating proximal tubules and in desquamated cells co-localize with the $\beta_1$ integrin subunit. Binding sites in the vasculature co-localize with the $\alpha_v$ subunit [Romanov V 1997].

The administration of cyclic RGD-peptides to rats following acute renal ischaemia, ameliorates the injury [Noiri E 1994]. To gain insight into the mechanism by which the amelioration occurs, effects of the peptides on cell:cell adhesion were delineated in-vitro,
using cultured BSC-1 cells. These peptides successfully blocked cell:cell adhesion, and it was therefore suggested that the beneficial action of the cyclic-RGD-peptides results predominantly from their ability to inhibit the cell:cell aggregation of sloughed desquamated cells and therefore reduce the severity of tubular obstruction [Noiri E 1995]. Osteopontin, which contains an RGD sequence, demonstrates increased expression in the distal tubule post-ischaemic renal injury. It has been proposed that the expression of RGD-containing osteopontin at distal nephron sites post-ischaemia could serve such a function following acute renal injury [Noiri E 1995, Padanilam BJ 1996, Romanov V 1997].

The expression of CD44 in cells of regenerating proximal tubules is observed within 1 day following injury (Figure 5.2, 5.3), as is the synthesis of hyaluronic acid in the peritubular interstitium (Figure 5.4). In contrast, osteopontin cannot be detected in regenerating proximal tubules until later (3 days post-ischemia) (Figure 5.5) suggesting that it may have some function in the recovery process of the kidney. The time course of osteopontin up regulation in the regenerating proximal tubules coincides with the period of re-epithelialisation of the tubular basement membrane. While CD44 and hyaluronic acid are not both localized in regenerating proximal tubule cells, CD44 and osteopontin are co-expressed in at least some cells (Figure 5.6). Such a temporal and spacial discordance between the synthesis of hyaluronic acid and osteopontin could reflect different roles for ligation of CD44 by each following ischaemic injury. For example, CD44:hyaluronic acid may mediate cell:matrix attachment and CD44-osteopontin co-expression could, in some way, regulate cell migration as it has been proposed to do in malignant tumour cells [Weber GF 1997].
The demonstration of de-novo CD44 and ligand expression post-ischaemia in the kidney are consistent with a key role for CD44:ligand interactions in the process of relining of the partially-denuded basement membrane by proliferating, migrating epithelial cells. We have shown that enhanced renal expression of one CD44 ligand, osteopontin, occurs in association with the amelioration of acute renal failure that results from the administration of insulin-like growth factor I to rats prior to induction of ischaemia [Miller SB 1994, Padanilam BJ 1996]. Our demonstration of CD44 expression in the kidney post-ischaemia sheds light on a possible mechanism for the beneficial action of IGF I in the rat [Miller SB 1992] and perhaps in humans [Franklin SC 1997], through the induction of osteopontin. A more thorough understanding of CD44:ligand interactions following renal injury in this setting could provide useful insights into additional therapeutic modalities.

A role for CD44 in tubule cell recovery was supported more recently by a study that demonstrated that following unilateral ureteral obstruction mice lacking CD44 developed more tubule damage, associated with decreased proliferation and increased apoptosis of tubule epithelial cells [Rouschop KMA 2004]. CD44 is predominantly expressed by damaged tubule cells and inflammatory cells in this model. It was proposed that CD44 expression may promote the maintenance of tubule cell viability in response to renal injury and that its interaction with osteopontin induces proliferation and decreases apoptosis of the tubule epithelial cell. Additionally macrophage clearance of apoptotic bodies is critical in the resolution of inflammation and failure to remove apoptotic cells may progress to secondary necrosis with damage to the surrounding tissue. CD44 has been implicated in the phagocytic clearance of apoptotic cells and therefore a lack of CD44 may contribute to increased tubular damage following injury [Teder P 2002].
CHAPTER 6

INHIBITION OF POLY (ADP-RIBOSE) POLYMERASE ATTENUATES ISCHAEMIC RENAL INJURY IN RATS

6.1 Introduction

Acute renal failure (ARF) continues to be the most costly kidney disease in hospitalized patients. At present there are no clinically accepted pharmacological interventions to attenuate cellular injury or augment tissue regeneration following ARF [Bonventre JV 1993, Conger J 1998, Star RA 1998]. To develop such a treatment, a fundamental understanding of the mechanisms of cellular injury and regeneration after the insult is essential. One means to acquire such an understanding is through the use of animal models of ischaemic injury [Chiao H 1998, Miller SB 1994].

Ischaemic renal injury results in damage to the most distal (S3) segment of the proximal tubule. The nature of the renal tubule epithelial cell injury depends upon the cell type and the severity of the injury to which the cell is exposed. A tubule epithelial cell may die or survive depending on the genetic programs that are activated [Bonventre JV 1993, Lieberthal W 1996]. Recent evidence indicate that depending on the severity of the injury, both necrosis and apoptosis play important roles in cell death immediately following ischaemic injury. The predominant form of cell death in ischaemic injury is by necrosis [Edelstein CL 1997, Venkatachalam MA 1978]. Several groups have provided evidence using electron microscopy and endonuclease mediated DNA fragmentation studies in in-vitro and in-vivo models of ischaemic renal injury that cell death secondary to apoptosis also occurs following renal ischaemia [Schumer M 1992, Shimizu A 1993, Ueda N 1992].

Apoptosis is also observed during the regeneration phase in the proliferating renal tubules following renal ischaemia [Padanilam BJ 1998]. Apoptosis at this stage may be important
to remove the excessive cells in the hyperplastic tubules and to maintain the tubules in their normal fully epithelialized state [Shimizu A 1993]. Dissecting and discriminating the molecular mechanisms of cell death via apoptosis and necrosis is important for defining how cell death is regulated in renal ischaemia and in developing rational strategies to modulate cell death for therapeutic purposes.

In models of ischaemic tissue injury, cellular DNA damage results from the generation of reactive radicals such as superoxide anions, hydrogen peroxide, nitric oxide or peroxynitrite, during tissue reperfusion. In the presence of single- or double-strand breaks in DNA, a chromatin-bound enzyme, poly (ADP-ribose) polymerase (PARP), transfers the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to nuclear proteins and to itself [Berger NA 1985, Hayaishi O 1997, Lindahl T 1995, Schraufstatter IU 1986]. PARP is an abundant, chromatin-bound enzyme constitutively expressed in numerous cell types. Proposed functions of PARP include stabilisation of V-shaped DNA conformations and facilitation of DNA access for various repair enzymes [Lindahl T 1995].

The activation of PARP results in a depletion of intracellular NAD. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP levels [Schraufstatter IU 1986, Satoh M 1992, Carson DA 1986]. Furthermore, nicotinamide formed by PARP activation can be recycled back to NAD via an ATP consuming pathway. Thus activation of PARP leads to a fall in ATP via two different mechanisms, which leads to cellular dysfunction and ultimately cell death. This process has been termed the “PARP Suicide Hypothesis”. Significant ischaemic injury that results in substantial DNA degradation requires that cells consume large amounts of ATP to support poly (ADP-ribosylation). For this reason, whereas a moderate activity of
PARP protects cellular genome integrity, its excessive activation can lead to cell death secondary to ATP depletion [Thiemermann C 1997, Zhang J 1994].

Ischaemic injury results in substantial DNA degradation and cells consume large amounts of ATP to support poly (ADP-ribosylation) reactions. Thus ATP is depleted leading to energy depletion and subsequent cell death [Berger NA 1985, Schraufstatter IU 1986]. Activation of PARP is a consequence of ischaemic injury in the brain, retina, heart and skeletal muscle [Lam TT 1997, Lo EH 1998, Takahashi K 1997, Thiemermann C 1997]. Several inhibitors of PARP including benzamide, 3-amino benzamide (3-AB) [Banasik M 1992] have been used to inhibit its activity in various models of ischaemic injury and neurotoxicity. Administration of PARP inhibitors have led to a significant reduction of infarct volume in a focal cerebral ischaemia model in the rat [Lo EH 1998, Takahashi K 1997], reduction in the infarct size caused by ischaemia-reperfusion of the heart or skeletal muscle in rabbits [Thiemermann C 1997] and ameliorated the ischaemia/reperfusion damage to the retina [Lam TT 1997]. During renal ischaemia, ATP levels fall to less than 25% of pre-ischaemic values and remain low for the duration of the insult. After the ischaemic episode reperfusion occurs during which ATP is required for cell regeneration and repair. The role that PARP plays in the process of recovery from the acute renal failure that follows ischaemic injury to the kidney is undefined. Whether PARP inhibition would curtail ATP loss or augment ATP recovery and thus ameliorate or protect the course of ischaemic renal injury has not been investigated.

In this study, we examined the hypothesis that induction of renal ischaemia may lead to the induction of PARP and may contribute adversely to the course of ischaemic renal injury. We evaluated the efficacy of administration of PARP inhibitors on renal function, cellular regeneration, ATP content and histopathology in rat models of ischaemic renal injury. Our
data indicate that a transient inhibition of PARP may be a novel approach for the therapy of acute renal failure.

6.2 Methods

Acute renal failure was induced by 60 minutes of bilateral renal artery clamping as described previously in Chapter 2. The animals were sacrificed at one or seven days post injury, and both kidneys were processed for histological analysis as before.

6.2.1 Administration of PARP inhibitors to rats

The PARP inhibitors benzamide and 3-amino benzamide were administered as described in Chapter 2.

6.2.2 Immunohistochemistry

Primary antibodies for PARP (Boehringer Mannheim or Enzyme systems products) were used for immunohistochemistry. The antibody was applied in one tenth concentration of the blocking buffer for 1 hour at room temperature.

6.2.3 PCNA analysis

Nuclear proliferating cell nuclear antigen (PCNA) was detected using a PCNA staining kit (Zymed, San Francisco, CA) as described in Chapter 2. Eight kidney sections originating from four different rats were viewed under X10 power lens, and the images from non overlapping outer medullary (S3) segments were captured on a computer. There was no significant difference in the levels of creatinine at 1 day post injury in either the vehicle-treated group (3.1± 0.42mg/dl) or the benzamide-treated group (2.7± 1.0 mg/dl) of the four rats used for histological analysis compared to the 16 rats that we used to generate the data in Figure 6.3.
6.2.4 Quantitation of tubule dilatation

The degree of morphological damage was assessed using sections of kidneys stained with haematoxylin and eosin as described in Chapter 2. Sections originating from kidneys of four vehicle- or benzamide-treated rats were evaluated. Levels of creatinine in neither the vehicle-treated group (2.9± 0.5 mg/dl) nor the benzamide-treated group (2.7± 0.5 mg/dl) of four rats, the tissues of which we used for histological analysis, differed significantly from the levels of creatinine at 1 day post injury in the corresponding groups of 16 rats that we used to generate the data shown in Figure 6.3.

6.2.5 Measurement of ATP content

The ATP content was measured as described in Chapter 2.

6.2.6 Western Blotting

Western blots were performed as described in Chapter 2. The blots were probed with the same monoclonal antibody for PARP as that used to perform immunohistochemistry.

6.3 Results

Prior to performing immunohistochemistry we demonstrated that the monoclonal mouse anti-PARP antibody recognised a protein in rat kidney with a molecular weight consistent with intact PARP. We performed Western blots of protein extracted from whole kidneys of sham-operated rats or rats previously rendered ischaemic (ARF) at 6 or 12 hours or at 5 days. No band is detectable in extracts of kidneys from sham-operated rats or rats rendered ischaemic 6 hours before extraction (Figure 6.1). However, a 116-kDa band in the extracts of kidneys of rats rendered ischaemic 12 hours or 5 days before extraction is of a size that would be predicted for intact PARP [Venkatachalam MA 1978].
Figure 6.1 Western blot of rat kidney extracts. Shown are results using extracts from whole kidneys of sham-operated rats or from kidneys of rats rendered ischaemic (ARF) 6 or 12 hours or 5 days before extraction. A single 116 kDa band is shown.
The spatial expression pattern of PARP following renal ischaemia was characterised by immunohistochemistry using the monoclonal mouse anti-PARP antibody. Shown in Figure 6.2 are sections of the damaged S3 segments of proximal tubules that are observed post-ischaemia in rat and corresponding sections from sham-operated controls. PARP immunoreactivity was not detected in sections originating from kidneys of rats 1 day after injury when control antibody was substituted for anti-PARP (Figure 6.2 A). PARP staining was not observed in sections originating from kidneys of sham-operated rats 1 day post-sham surgery (Figure 6.2 B). No PARP immunoreactivity was observed in proximal tubular cells at 6 hours (Figure 6.2 C) post-ischaemia. However positive staining was observed at 12 hours (Figure 6.2 D), 1 day (Figure 6.2 E) and 5 days (Figure 6.2 F) after injury. PARP was localised to nuclei of cells in the S3 segment of the proximal tubule, in cells sloughed into the tubule lumen (Figures 6.2, D and E), and in papillary proliferations (Figure 6.2 F).
Figure 6.2 Immunohistochemical localisation of poly (ADP-ribose) polymerase (PARP) in ischaemia-injured kidneys. Shown in A is a section from a kidney rendered ischaemic 1 day before death, stained using control antibody. Also shown is expression of PARP protein in kidneys from sham-operated rats (B) and rats rendered ischaemic 6 hours (C), 12 hours (D), 1 day (E), or 5 days (F) before death. Arrowheads show positively staining cells. p, Papillary proliferation. Magnification is shown in F.
To elucidate whether inhibition of PARP activity ameliorates the course of ischaemic renal injury, we investigated the effects of administering each of the PARP inhibitors Benzamide or 3-amino Benzamide, to rats rendered ischaemic. The levels of serum creatinine (Figure 6.3) and blood urea nitrogen (BUN) (Figure 6.4) were no different at 24 hours after injury in animals that received either benzamide or 3-AB compared with the levels in vehicle-treated rats. However, creatinine was significantly reduced during days 2-5 post-ischaemia and the BUN values at days 2-5. The mortality rate among the vehicle-treated and benzamide-treated rats was identical. In both cases, two animals out of eighteen died within 24 hours post-injury. There was no mortality among the five rats that were treated with 3-AB. No change in the levels of creatinine or BUN were detected over a 6-day time period among sham-operated rats that received vehicle, benzamide, or 3-AB (data not shown).
Figure 6.3 Effect of PARP inhibitors on serum creatinine. Comparison of the effects of the PARP inhibitors, benzamide (Benz) or 3-amino benzamide (3AB), on the levels of serum creatinine following ischaemic injury or following administration of vehicle. The values at each time represents means ± SE. The values at days 2-5 are significantly different (*P < 0.05).
Figure 6.4 Effect of administration of PARP inhibitors, benzamide or 3-amino benzamide, on the levels of blood urea nitrogen (BUN) compared with that from vehicle-treated rats. The values at each time point represent means ± SE. The values at days 2-5 are significantly different (*P < 0.05).
Histological analysis of kidney sections obtained from rats that were treated with vehicle or one of the PARP inhibitors, benzamide, seven days post-ischaemic injury, showed that both groups suffered ischaemic damage. Shown in Figure 6.5 A is a section from a kidney originating from a vehicle-treated rat stained with haematoxylin and eosin. A section of a kidney from a benzamide-treated rat is shown in Figure 6.5 B. Kidneys from animals that received benzamide had fewer dilated tubules compared with kidneys from a rat that received vehicle (Figure 6.5 C).

PCNA expression was assessed immunohistochemically in kidneys from vehicle- or benzamide-treated rats obtained 24 hours post-ischaemic injury. PCNA-positive cells were found both in the cortex and in the outer medullary segments in kidneys from the vehicle- or benzamide-treated rats. Shown in Figure 6.5 D is a section from a kidney originating from a vehicle-treated rat stained for PCNA. A section from the benzamide-treated rat shows more PCNA-positive cells in a comparable area (Figure 6.5 E). Kidneys originating from vehicle-treated rats had a relatively lower number of PCNA-positive cells in the cortex and outer medullary segments (Figure 6.5 F).
Figure 6.5 Effect of administration of the vehicle (A,D) or PARP inhibitor benzamide (B,E) on renal histopathology 7 days post injury (A-C) and proliferation (D-F) 1 day post-injury. A and B show haematoxylin and eosin stained sections. D and E show proliferating cell nuclear antigen (PCNA)-stained sections. Arrowheads show PCNA-positive nuclei (D and E).
To determine whether PARP inhibition increased levels of renal ATP post-ischaemia, kidneys from vehicle-or benzamide-treated rats that underwent 60 minutes of ischaemia followed by reperfusion for 24 hours were isolated and the ATP contents were determined. At 24 hours post-ischaemia, ATP levels in kidneys rendered ischaemic would be expected to be approximately 60 percent of normal [Karasaswa A 1990].

For our studies, kidneys derived from sham-operated rats served as controls. At 24 hours post-ischaemia in vehicle-treated rats, ATP levels were significantly reduced (P < 0.05) to 54% (0.683± 0.064 nmol/mg protein) of those in kidneys of sham-operated rats (1.256± 0.157 nmol/mg protein). Levels of ATP in benzamide-treated rats that were rendered ischaemic were significantly (P < 0.05) greater (85%) than those in vehicle-treated controls (1.073± 0.273 nmol/mg protein), consistent with preservation of ATP content post-ischaemia by benzamide.

6.4 Discussion
Ischaemic injury of the kidney results in the generation of reactive oxygen species such as the superoxide radical and its reduction products hydrogen peroxide and the hydroxyl radical [Ueda N 1992]. In addition, the inducible form of nitric oxide synthase (iNOS) is activated, and nitric oxide is generated post-ischaemic injury. Nitric oxide reacts with superoxides to form peroxynitrite. A role for the reactive oxygen species and peroxynitrite in mediating renal damage has been substantiated by the observation that pretreatment of rats with free radical scavengers and inhibition of iNOS ameliorate the course of ischaemic injury [Noiri E 1999, Peresleni T 1996].

There is evidence that reactive oxygen species and peroxynitrite induce cellular injury by inducing nicks in DNA. DNA damage is repaired via the activity of several DNA repair
enzymes, including PARP [Hagar H 1996, Schraufstatter IU 1986, Shah SV 1992, Ueda N 1998]. PARP activation occurs in the settings of cerebral, cardiac and skeletal muscle ischaemia. The data are shown in Figures 6.1 and 6.2 demonstrate that PARP is also induced after ischaemic injury of the kidney in the S3 proximal tubule segment (Figure 6.2).

PARP maintains genome integrity after cellular exposure to genotoxic agents in the setting of ischaemic injury. However, extensive DNA damage after ischaemic injury may lead to excessive PARP activation that consumes a large quantities of cellular NAD, resulting in ATP depletion and death [Zhang J 1994]. Therefore, it has been proposed that, although chronic inhibition of the activity of PARP is likely to be harmful to the cell, transient inhibition after ischaemic injury may prevent cell death [Thiemermann C 1997].

Our data showed that transiently inhibiting PARP activity after renal ischaemia/reperfusion injury accelerates recovery, as reflected by lower levels of creatinine and BUN during days 2-5 post-ischaemia (Figures 6.3 and 6.4) and improved renal histology at seven days after injury (Figure 6.5). Inhibiting PARP activity lead to an increase in the number of regenerating cells at 24 hours post-injury (Figure 6.5), consistent with acceleration of the tubule repair process.

We cannot exclude the possibility that the data are in Figure 6.3 and 6.4 reflect actions of PARP inhibition to reduce BUN generation and creatinine release in the setting of acute ischaemic renal injury. However, the data are shown in Figure 6.5, A-F, considered together with those shown in Figures 6.3 and 6.4 and our finding that neither inhibitor affects levels of creatinine or BUN in sham-operated rats over a period of seven days, render it likely that the reductions in creatinine and BUN (Figures 6.3 and 6.4) reflected an
amelioration of ischaemic injury. Inhibition of PARP after renal ischaemia did not affect levels of creatinine or BUN measured at 24 hours post-ischaemia (Figures 6.3 and 6.4). This finding could indicate that the extent of the initial renal damage sustained was not affected by inhibiting PARP. Alternatively, an amelioration of injury affected by PARP inhibition may be inadequately reflected by measurements of creatinine and BUN so soon after injury but maybe better reflected by the higher ATP levels demonstrated in treated rats post-injury.

The regenerative capacity of the renal proximal tubule immediately after ischaemic injury is dependent on the number of non-injured or sublethally injured tubule epithelial cells that survive and can initiate the reparative mechanisms that restore the structure and physiological function of the renal tubule epithelium [Bonventre JV 1993, Lieberthal W 1998]. One of the major requirements for the initiation of the cellular repair process is the repletion of intracellular ATP. ATP levels fall to undetectable levels after 60 minutes of renal ischaemia. During the first two hours after the ischaemic insult, ATP recovery occurs in two phases. There is a rapid initial increase in levels of ATP that occurs immediately on reflow followed by a more gradual elevation to normal levels [Stromski ME 1986, Weinberg JM 1991].

The restoration of ATP levels back to normal takes greater than 48 hours [Karasawa A 1990]. Our data showing that PARP inhibition post-injury restores levels of ATP close to normal levels at 24 hours are consistent with one of the mechanisms by which PARP inhibition ameliorates the course of injury being preservation of ATP levels.

It is of interest, that at seven days post-ischaemia, renal cortices from benzamide-treated rats appear more normal than those of vehicle-treated rats, whereas at the same time, there
are no significant differences in serum creatinine or BUN (Figures 6.3 and 6.4). Such a disassociation between histology at seven days after renal ischaemia and levels of creatinine and BUN was previously observed in rats treated with insulin-like growth factor-I (IGF-I) or epidermal growth factor compared with the vehicle-treated rats [Miller SB, 1992, 1994] and a similar disassociation between disturbances of structure and function is well described in human acute renal failure [Lieberthal W 1998].

We have shown that PARP is expressed in the damaged S3 segments of the renal proximal tubule beginning within 12 hours of renal injury and that transient inhibition of PARP activity post-ischaemia ameliorates the course of acute renal failure. In vitro work has now been published to support our findings using PARP inhibitors to protect rat proximal tubular cells against oxidant stress [Chatterjee PK 1999]. This study proposed that PARP activation contributes to reactive oxygen species-mediated injury of rat proximal tubule epithelial cells.

As is the case for other agents with similar effects on the course of renal injury [Miller SB, 1992, 1994], such as IGF-I, the exact mechanism by which PARP inhibition is salutary remains undefined and may reflect direct or indirect actions on renal tissue. However, whatever the basis for their beneficial action may be, our findings provide a rationale for the development and pharmacological use of suitable inhibitors of PARP to accelerate recovery from acute renal failure in humans.

It is necessary to proceed with caution along any such pathway. As in certain circumstances, inhibition of PARP may be harmful because this enzyme facilitates DNA repair that may be required in the recovery phase of acute tubular necrosis. For this reason,
determination of the proper timing for PARP inhibition post-injury may be a crucial factor in the administration of PARP inhibitors.
CHAPTER 7

FUTURE DEVELOPMENTS IN ISCHAEMIC ACUTE RENAL FAILURE

7.1 Introduction

It is unfortunate that despite a considerable amount of scientific research, which has provided us with a much greater understanding of the pathophysiology of acute renal failure (ARF), little progress has been made in the treatment of this condition. Studies have been very successful in elucidating the vascular and tubule epithelial cell abnormalities that are involved in the pathogenesis of ARF, allowing the successful use of the pharmacological interventions in ameliorating the renal dysfunction in animal models [Chiao H 1997, Miller SB 1992, 1994]. However translating such promising results from animals into successful clinical studies in patients with ARF has been less successful [Hirschberg R 1999]. There is therefore much work still to be done to try and better understand the complex pathogenesis of ARF. Since the completion of my studies into gene expression following ischaemic renal injury a number of advances in scientific research have been made including the sequencing of the human genome, which along with other discoveries may yet provide us with the knowledge necessary to develop effective therapeutic interventions.

7.2 Diagnosis of acute tubular necrosis

The traditional blood and urine markers for the diagnosis of various renal diseases are insensitive and nonspecific. In acute tubular necrosis (ATN) the ability to identify the development of tubule epithelial cell damage is in important factor in the administration of potential pharmacological interventions. It has been demonstrated in animal models that the success of therapies is dependent on the time of administration with respect to the onset
of ischaemic injury. Kidney Injury Molecule-1 (KIM-1) is a type 1 transmembrane protein, with an immunoglobulin and mucin domain, whose expression is markedly upregulated in the proximal tubule following ischaemic renal injury in the rat [Ichimura T 1998]. A soluble form of human KIM-1 is detectable in the urine of patients with ATN, and it has been proposed that it may serve as a useful biomarker facilitating the early detection of proximal tubule cell injury [Han WK 2002].

7.3 Clinical investigation of acute tubular necrosis

In ATN tubule epithelial cell damage coexists with altered glomerular haemodynamics. The relative contribution of each of these factors to the development of renal dysfunction has yet to be fully established. Our understanding of the pathogenesis of ATN in humans has been limited by a lack of noninvasive techniques to assess the renal parenchymal microcirculation and cellular energy depletion. There are now improved functional imaging techniques such as blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI), which takes advantage of the capacity of deoxyhaemoglobin to act as an endogenous contrast agent [Prasad PV 1996]. This noninvasive technique has been used to examine regional alterations in the oxygenation of the kidney in response to a variety of physiological and pathophysiological perturbations such as intravenous radiocontrast administration [Prasad PV 2001]. Such a technique could in the future be used to gain clinical data on the changes in renal parenchymal oxygenation during the development of ATN.

7.4 The development of functional genomics and proteomics

Upon the completion of the Human Genome Project one of its stated future goals was the development of functional genomics [Collins SC 1998]. The development of high-density microarray or gene chip technology has provided a powerful tool to investigate gene
expression [Ramsay G 1998, Schena M 1998]. Microarray-based studies are capable of uncovering broad patterns of genetic activity, providing new understanding of gene functions and generating insight into transcriptional processes and biological mechanisms. Gene chip technology has now been used to start investigating changes in gene expression following ischaemic renal injury in animal models [Yoshida T 2002]. Clinical studies could study gene expression patterns in renal biopsy samples using laser microdissection [Kretzler M 2002]. Laser microdissection allows gene expression analysis of circumscribed nephron segments [Kohda Y 2000, Murakami H 2000]. It offers the unique opportunity to correlate structural information with gene expression programs on the same tissue sample. This can in turn be related to clinical information available on the biopsy samples.

The development of functional proteomics has now provided the opportunity to study protein expression in a similar fashion to those of gene expression. Recent work has used proteomic technology to construct protein maps of rat kidney cortex and medulla [Arthur JM 2002]. In this study differential expression of proteins between the cortex and medulla was demonstrated. Proteomics should provide a useful tool for detecting changes in renal protein expression following ischaemic renal injury. Such studies should provide new data on the pathogenesis of the ARF and provide potential targets for therapeutic intervention.

7.5 Future potential therapeutic interventions

Recovery of renal function following ATN is dependent upon the replacement of necrotic tubule cells with a functional epithelium. The discovery that extrarenal cells (haemopoietic stem cells) may play a role in the regenerative process following acute renal failure provides a rationale for the cellular therapy of acute renal failure [Gupta S 2002, Lin F 2003]. However, it is important to establish that these repopulating extrarenal cells not
only resemble the indigenous population, but also share their functional competence. This work remains in its infancy but may provide a new avenue for exploring a novel therapeutic strategy for clinical acute renal failure.

Of recent interest in the kidney is the phenomenon of ischaemic preconditioning [Bonventre JV 2002]. Ischaemic preconditioning was first described in the heart and its effect has been reported to be greater than the protective effects afforded by pharmacological interventions in animals. Ischaemic preconditioning renders the mouse kidney resistant to subsequent ischaemia [Park KM 2003]. Studies have therefore been embarked on to further understand the endogenous processes that the kidney has developed to protect itself against an ischaemic insult. This may provide a further avenue for the development of new therapeutic interventions for acute renal failure.
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