Hypoxia is a pro-fibrogenic stimulus for human renal tubular epithelial cells

by

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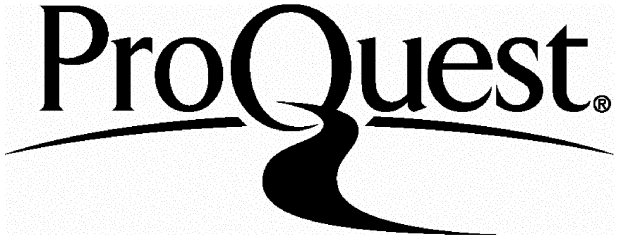

Abstract

Progressive renal diseases of diverse etiology are characterised by tubulo-interstitial fibrosis which ultimately leads to renal failure. One important feature of TIF is thickening of the tubular basement membrane with altered matrix composition. The underlying mechanisms which mediate the qualitative and quantitative changes in the TBM have not been identified; however one initiating stimulus may be hypoxia arising from microvascular injury. Since hypoxia is a potent regulator of gene expression it was hypothesised that oxygen deprivation may provide a stimulus for changes in ECM metabolism in PTE.

The effects of hypoxia on PTE matrix synthesis and degradation were examined using an *in vitro* model. In human PTE, hypoxia (1%O_2, 24hrs) increased total collagen production, decreased MMP-2 actvity and increased Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) protein. Collagen IV mRNA levels decreased while collagen α1(I) mRNA increased, suggesting induction of an atypical, interstitial collagen. Hypoxia-induced changes persisted on re-oxygenation, with increased expression of TIMP mRNAs. Neutralising antibody studies demonstrated that these effects were not due to the induction of the major pro-fibrogenic cytokine TGF-β1. Moreover, the hypoxia-induced increase in collagen α1(I) was not mediated by a secreted, soluble factor, suggesting a direct effect of hypoxia on the collagen α1(I) gene. Transient-transfection experiments demonstrated that collagen α1(I) promoter activity is stimulated by hypoxia and that this effect is independent of HIF-1, the transcription factor which regulates transcription in many hypoxia-responsive genes. This suggests an alternative mechanism for regulation of the collagen α1(I) promoter by hypoxia.

In summary, this thesis demonstrates that hypoxia is a pro-fibrogenic stimulus for PTE *in vitro* simultaneously increasing matrix production and decreasing turnover. Similar effects of hypoxia *in vivo* would lead to the qualitative and quantitative changes observed in TIF.

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.

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In memory of

Fred Wilson

1932-1999

CHAPTER 1

Introduction

1.1. Rationale

Progressive renal diseases, characterised by accumulation of extracellular matrix (ECM) and expansion of the tubulo-interstitial compartment, culminate in renal failure and therefore represent a significant clinical problem [1] Although these diseases are diverse in etiology [2](*Table 1.1*), their common feature is marked tubulo-interstitial fibrosis (TIF), characterised by tubular basement membrane (TBM) thickening, accumulation of interstitial ECM, tubular dilatation and atrophy, accompanied by loss of the microvasculature [3-5].

Traditionally, studies of progressive renal disease focused on the glomerular compartment since many of these diseases are thought to be glomerular in origin; however it is now recognised that the severity of TIF remains the best prognostic indicator of disease progression and unlike glomerular injury, directly correlates with glomerular filtration rate [6-8]. The association between TI involvement and disease progression has recently focused attention on this compartment in an attempt to understand the pathogenesis of chronic renal failure.

To date the mechanisms underlying TIF have not been identified [5]. It remains unclear why, even in diseases where the initial insult is well-defined (eg. acute drug-induced tubulotoxic injury), TIF persists despite removal of the injurious stimulus [9-10]. Thus it would appear that once TIF is triggered, a cycle or cascade of events ensure that it is progressive and unrelenting, ultimately resulting in organ failure [10]. One potential initiating stimulus for fibrogenesis is hypoxia. Microvascular damage resulting from glomerular injury is a common feature of progressive renal diseases and, presumably,

WHO Classification of tubulointerstitial diseases
Infection:
Acute infectious tubulointerstitial nephritis
Acute tubulointerstitial nephritis associated with systemic infection
Chronic infectious tubulointerstitial nephritis (chronic pyelonephritis)
Specific renal infection
Drug-induced tubulo-interstitial nephritis:
Acute drug-induced tubulotoxic injury
Drug-induced hypersensitivity tubulointerstitial nephritis
Chronic drug-induced tubulointerstitial nephritis
Tubulo-interstitial nephritis associated with immune disorders:
Induced by:
antibodies reacting with tubular antigens
autologous or exogenous antigen-antibody complexes
cell mediated hypersensitivity
immediate hypersensitivity
Obstructive uropathy
Vesicoureteral reflux associated nephropathy (reflux nephropathy)
Tubulointerstitial nephritis associated with papillary necrosis
Heavy-metal induced tubular and tubulointerstitial lesions
Acute tubular injury/necrosis:
Toxic
Ischaemic
Tubular and interstitial nephropathy caused by metabolic disturbances
Hereditary renal tubulointerstitial disorders
Tubulointerstitial nephritis associated with neoplastic disorders
Tubulointerstitial lesions in glomerular and vascular diseases
Miscellaneous disorders:
Balkan nephropathy

Table 1.1 *WHO Classification of tubulointerstitial disease.* Adapted from [2].

results in impaired O_2 delivery to the tubulo-interstitum [11,12]. Due to their high metabolic activity, proximal tubular epithelial cells (PTE) are particularly sensitive to O_2 deprivation and are potentially a target cell for a hypoxic insult [13,14]. Since, hypoxia is a potent regulator of gene expression [15], it was hypothesised that O_2 depletion may alter ECM metabolism in PTE, potentially acting as a pro-fibrogenic stimulus for this cell type. Ischaemia may therefore provide the crucial link between glomerular damage and TIF, representing a common stimulus for ECM accumulation by PTE in progressive diseases of diverse origin.

1.2 Background

1.2.1 The tubulo-interstitium

In order to understand the tubulo-interstitial changes which occur in fibrosis, it is first necessary to consider the normal tissue architecture. The human kidney contains approximately 1 million nephrons; each nephron being a single filtration unit [16] (*Fig. 1.1.A and B*). The proximal tubule is that segment of the nephron immediately adjacent to the glomerulus within the cortex and is basally surrounded by interstitial fibroblasts, peritubular capillaries and resident macrophages [17]. These different cell-types together with the sparse interstitial ECM (*Fig. 1.2.A*) is collectively known as the cortical tubulo-interstitium (TI) [17].

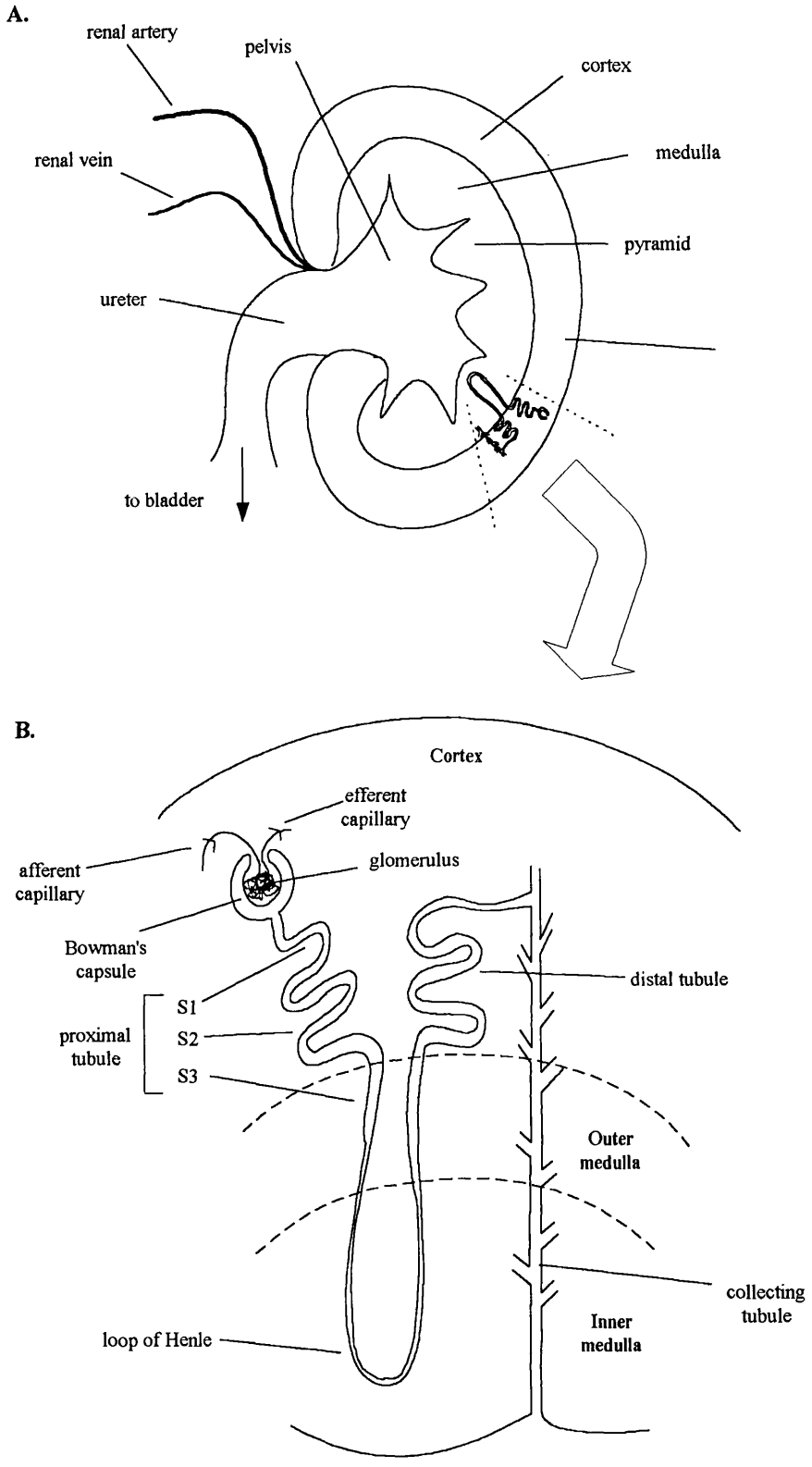


Fig. 1.1 ***Structure of the human kidney.*** **A**. Cross section of kidney illustrating the position of nephrons relative to the whole kidney. **B**. Single nephron indicating position of proximal tubule within the cortex (adapted from [18,19]).

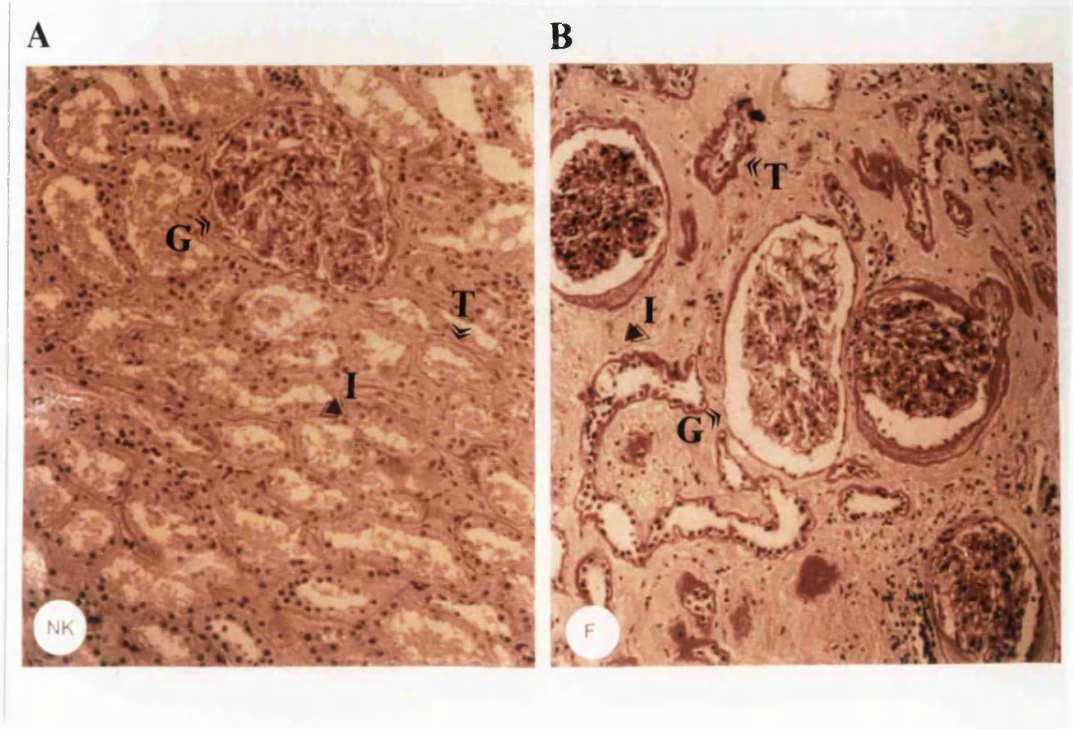


Fig. 1.2 ***The normal and fibrotic cortical-tubulo-interstitium.*** **A.** Cross-section of normal kidney (**NK**) tissue showing tubules (**T**) and glomeruli (**G**) within a sparse interstitial ECM (**I**). **B.** Cross-section of tubulo-interstitium in fibrosis (F) showing accumulation of interstitial ECM and thickened tubular basement membrane.

The proximal tubule is divided into three distinct segments: S1 and S2, which constitute the convoluted tubule and S3, the straight portion [18] (*Fig 1.1.B*). Although the cells of these three regions have unique ultrastructural features, the proximal tubule epithelium is a polarized epithelium, structurally and functionally specialised for selective reabsorption and secretion of ions, water and macromolecules [20]. A number of features allow PTE to undertake their transport functions; the apical brush border is well-developed with densely packed microvilli and extensive invaginations at the basolateral surface ensure maximal surface area for reabsorption and secretion [13-18] (*Fig 1.3*). Close to the apical membrane, a complex vacuolar and lysosomal compartment allows non-specific reabsorption and degradation of macromolecules [13] while active transport of molecules across the basolateral surface is fuelled by the presence of many large, elongated mitochondria [13]. In addition, tight junctions

positioned at the intersection of apical/basolateral plasma membranes selectively regulate the passage of ions between cells and preserve polarity by limiting the diffusion of apical and basolateral membrane constituents [20]. These ultrastructural features ensure that appropriate vectorial transport is maintained and that the large energy requirements of these cells are met.

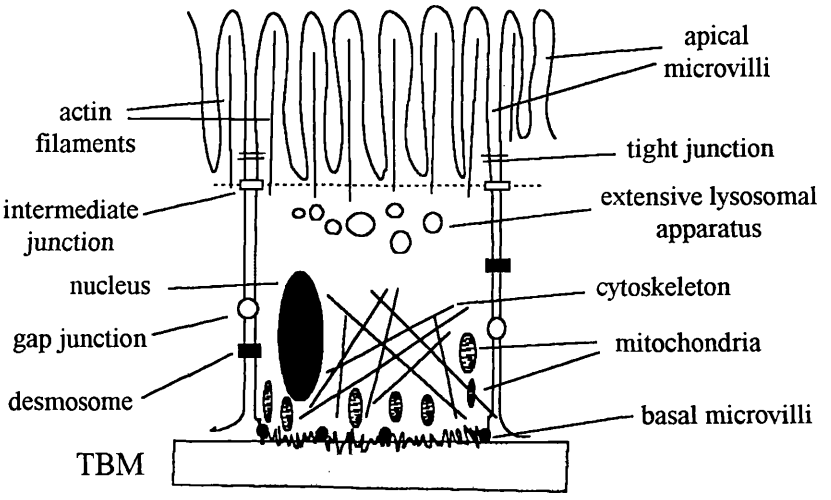


Fig. 1.3 ***Schematic diagram of a proximal tubular cell.*** TBM; tubular basement membrane (adapted from [13, 18]).

PTE are separated basally from adjacent interstitial cells and the microvasculature by a specialised TBM [21] composed predominantly of collagens IV and V, laminins, nidogen, fibronectin, heparan sulphate proteoglycans and glycoproteins [22]. In addition to providing structural support and creating an anatomical barrier restricting the flow of molecules and the migration of cells, the TBM serves as a modulator of cell behaviour and cell interaction and as a reservoir for cytokines and growth factors [23]. The importance of the TBM composition is clearly demonstrated in the marked differences in cell behaviour and response to stimuli when PTE are plated on different extracellular matrices *in vitro* [24].

The close apposition of capillaries to the TBM allows efficient reabsorption of water and solutes from the proximal tubule back into the circulation whilst providing the cells with the necessary O_2 supply for their functional needs [25]. Interstitial fibroblasts are found distributed throughout the interstitial compartment, their long cellular processes contacting adjacent cell types and providing a bridge for endothelial-epithelial cell interaction [17]. A sparse ECM, rich in collagens I and III surrounds the different cell types of the tubulo-interstitium, providing physical support and acting as a storage site for cytokines, growth factors and other molecules [26]. The tubulo-interstitium is therefore a dynamic entity, with closely apposed cell types in continuous communication, via cell-cell and cell-matrix interactions, to maintain homeostasis. Disruption of these paracrine interactions has profound consequences on cell function [27].

1.2.2 Tubulo-interstitial fibrosis

In fibrosis, the histology of the tubulo-interstitial compartment is grossly altered with all of the component cell types undergoing changes which collectively lead to destruction of normal kidney architecture and loss of nephron function [1]. Irrespective of the initiating disease, examination of tissue sections from fibrotic kidneys show an accumulation of ECM both within thickened TBM and in the expanded interstitial area [3-5](*Fig 1.2.B*). Proximal tubules may undergo atrophy whilst others appear dilated, possibly as a consequence of hyperfiltration in remaining tubules [7]. Another common feature is loss of the microvasculature; post-glomerular capillaries often appear collapsed or constricted and arteriolar walls may undergo sclerosis, thereby restricting O_2 delivery to the tubulo-interstitium [11,12,28]. The expansion of the interstitium in fibrosis is largely attributed to increased production of ECM proteins by fibroblasts which accumulate at the site of injury [5]. In addition, fibroblasts undergo 'activation' to

become myofibroblasts, expressing α-smooth muscle actin (α-SMA) and synthesising large amounts of ECM material [29]. While in normal wound healing, the appearance of myofibroblasts is transient, in fibrosis, this contractile phenotype persists, participating in the contraction of fibrotic scar tissue [30].

Although histologically it can be difficult to determine the initial stimulus and precise sequence of events which lead to the fibrotic phenotype, 3 phases of tubulo-interstitial disease are generally recognised [26]. Firstly, in response to an initiating insult, tubules are damaged and inflammatory cells migrate to the site of injury; monocytes, macrophages and lymphocytes accumulate, releasing a vast array of cytokines and growth factors [4]. This is followed by a complex cascade of cell-cell and cell-matrix interactions which results in the accumulation of ECM proteins within the TBM and the interstitium [27, 31, 32]. In diseases which regress, such as acute tubular nephritis, tubular regeneration restores nephron function [33,34]; however in progressive disease a relentless, scarring continues until complete tubular atrophy, microvascular loss and contraction of myofibroblasts create the small, highly compacted, non-functioning kidney that characterises end-stage renal failure [1,10].

1.2.3 Role of PTE in TIF

Although the stimuli which trigger TIF remain unknown, the observation that tubular injury is an early indicator of disease suggests that proximal tubular cells may be a primary target [7,35]. PTE are a rich source of cytokines and growth factors which can act in an autocrine or paracrine manner to influence almost every aspect of cell behaviour including cell motility, cell proliferation, ECM metabolism and the regulation of other growth factors and cytokines [27,36,37]. Studies investigating the inflammatory phase of progressive renal disease have shown that PTE can produce a number of

chemokines which may attract immune cells to the site of injury to initiate inflammation [38-41]. PTE derived from fibrotic human kidneys show increased mRNA expression for the chemoattractant, Granulocyte and Macrophage Colony Stimulating Factor (GM-CSF) [37] whilst in an animal models of fibrosis, PTE have elevated levels of monocyte chemoattractant protein-1 (MCP-1) [38] and the chemoattractant protein, RANTES (Regulated-Upon-Activation-Normal-T-cell-Expressed-and-Secreted) [39]. In some models of disease, PTE can also express the major histocompatibility complex (MHC) class II molecules on the cell surface providing a mechanism by which tubular cells can be directly involved in antigen presentation to lymphocytes [40,42].

In addition to pro-inflammatory molecules, PTE produce a number of pro-fibrogenic cytokines capable of inducing the synthesis of ECM proteins [36,43]. These can act in an autocrine manner to modulate production of BM components or regulate ECM proteins in adjacent cell types [44-46]. In TIF, the expansion of the TBM is mainly attributed to an increase in synthesis of collagens IV and V, laminin and heparan sulphate proteoglycans [47-49]. Interestingly, in addition to increased basement membrane components, fibrotic PTE show increased mRNA expression for the interstitial collagen, collagen I [50] and *in vitro* models of fibrosis demonstrate that PTE have increased levels of the interstitial matrix proteins, collagen I and fibronectin [51,52]. This induction of 'abnormal' matrix proteins is largely attributed to Transforming Growth Factor-β1 (TGF-β1) [53]. TGF-β1 is the premier fibrogenic cytokine [54-56], it is produced by PTE and expression is elevated in many experimental models of tubulo-interstitial fibrosis such as acute puromycin aminonucleoside nephrosis (PAN) [57] and protein-overload proteinuria [38] and human diseases including chronic glomerulonephritis and diabetic nephropathy [58,59]. In one *in vitro* model, a rat tubular epithelial cell line exposed to TGF-β1 showed an 8-fold increase in mRNA levels of

collagen I [44], suggesting a role for this cytokine in the upregulation of atypical proteins within the fibrotic TBM. In addition to stimulating production of matrix proteins, TGF-β1 is known to be anti-proliferative for most epithelial cell types [60] including PTE [61] and it has been suggested that an autocrine mechanism may exist which further injures the epithelial cell by preventing regeneration after a fibrotic insult [27]. Furthermore, TGF-β1 released by PTE can potentially act in a paracrine manner to modulate the behaviour of adjacent fibroblasts, endothelial cells and macrophages.

In addition to TGF-β1, PTE produce a number of other growth factors whose expression is altered in fibrosis [36,37]. PTE are a rich source of PDGF and tubular epithelial cells derived from fibrotic kidneys express higher levels of PDGF-B mRNA and protein than cells from control kidneys [37]. PDGF is known to be a potent mitogen for fibroblasts [46] and a paracrine growth loop has been demonstrated for epithelial and fibroblastic cells in human kidney cortex; PTE grown in co-culture with cortical fibroblasts release PDGF which stimulates fibroblast proliferation [46]. PTE also produce endothelins-1 and -2 (ET-1, ET-2) [62] and an increase in ET-1 has been demonstrated in PTE treated with high density lipoprotein (HDL) *in vitro* (to mimic the high levels of HDL found in the urine of nephrotic patients) [63]. Studies in our laboratory have demonstrated an autocrine growth loop for ET-1 in PTE; secreted ET-1 binds to the ET_B receptor on the cell surface to induce PTE proliferation [62]. Moreover, ligand/receptor binding itself can regulate ET-1 synthesis [62]. ET-1 also has paracrine effects; it acts as a vasoconstrictor on endothelial cells [64] and induces proliferation and matrix synthesis in cortical fibroblasts [65], illustrating another way how molecules produced by PTE in fibrosis can influence the surrounding cell types.

1.2.4 Matrix turnover and tubulo-interstitial fibrosis

The excessive deposition of ECM in fibrosis is thought to arise not only through an increase in synthesis but also through decreased degradation [66]. Two main pathways for ECM degradation have been described in the context of tubulo-interstitial fibrosis: the plasmin-dependent cascade and the matrix metalloproteinase pathway (MMP) [5]. In the plasmin-dependent system, the serine protease plasmin is converted from its inactive circulating precursor form, plasminogen, to its catalytic form, plasmin, by the action of the activating enzymes, urokinase-plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) [67]. Once active, plasmin can directly degrade both components of interstitial connective tissues and basement membrane while activating other matrix-degrading enzymes and releasing bound growth factors from the ECM [5]. Plasmin action is inhibited by α_2-anti-plasmin (α_2-AP) whilst the two main inhibitors of plasminogen activators are plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) [68]. The involvement of the plasmin cascade in tubulo-interstitial fibrosis has not been fully investigated, however increased PAI-1 expression by PTE, is a common feature of experimental models of interstitial fibrosis such as PAN nephrosis in rats [5] and protein-overload proteinuria in mice [38].

The majority of studies to examine the degradation of the ECM in tubulo-interstitial fibrosis have focussed on the actions of the matrix metalloproteinases (MMPs or matrixins). The MMPs are a family of zinc-containing proteases which act at neutral pH and together can degrade all the components of the ECM [69]. The majority of these enzymes are secreted as soluble, latent pro-enzymes which require activation for proteolytic activity [69]. To date, 13 mammalian MMPs, with overlapping substrate specificity have been identified; these include the gelatinases, collagenases, stromelysins and the membrane-type MMPs (MT-MMPs) [70] (*Table 1.2*).

Subgroup	Common name	MMP number	Molecular species (latent)	Substrates
Interstitial collagenases	Interstitial collagenase (fibroblast type)	MMP-1	52,57kD	Fibrillar collagens (III>>I)
	PMN collagenase	MMP-8	75kD	Fibrillar collagens (I>>III)
	Collagenase-3	MMP-13	54kD	Fibrillar collagens
Stromelysins	Stromelysin-1	MMP-3	52,58kD	LMN, FBN core protein, other MMP non-helical coll
	Stromelysin-2	MMP-10	58kD	Same as MMP-3
	Matrilysin	MMP-7	28kD	Similar to MMP-3
Gelatinases	Gelatinase-A	MMP-2	72kD	Gel, col IV, V
	Gelatinase-B	MMP-9	92kD	Gel, col IV, V
Elastases	Metalloelastase	MMP-12	53kD	Elastin
RXKR secreted type	Stromelysin-3	MMP-11	29kD	α-1-antitrypsin
RXKR membrane type	MT-MMP-1	MMP-14	66kD	Progelatinase-A
	MT-MMP-2	?	76kD	?
	MT-MMP-3	?	70kD	Progelatinase-A

Table 1.2 ***MMPs and their substrates.*** (Adapted from [70]).

All MMPs possess a catalytic domain which contains a zinc-binding region and a propeptide domain that is cleaved during activation [69] (*Fig.1.4*). Activation is thought to occur via a 'cysteine switch' mechanism, whereby attachment of the zinc-binding region of each molecule to a cysteine residue regulates enzyme state [71]. The cysteine residue remains cryptic ('closed conformation') in the latent form of the enzyme but is exposed during activation as a result of conformational change ('open

conformation') [69]. Once in the 'open' form, MMPs undergo a series of autocatalytic cleavages to remove the pro-peptide domain and generate the fully-processed active enzyme [69]. There is little information regarding the activation process *in vivo*; in the case of MMP-9, the enzymes plasmin, neutrophil elastase and cathepsin G have been implicated as physiological activators but these endopeptidases have no effect on MMP-2 [72]. Instead, activation of MMP-2 appears to be cell membrane-mediated with MT1-MMP cleaving the latent propeptide to its active form [73].

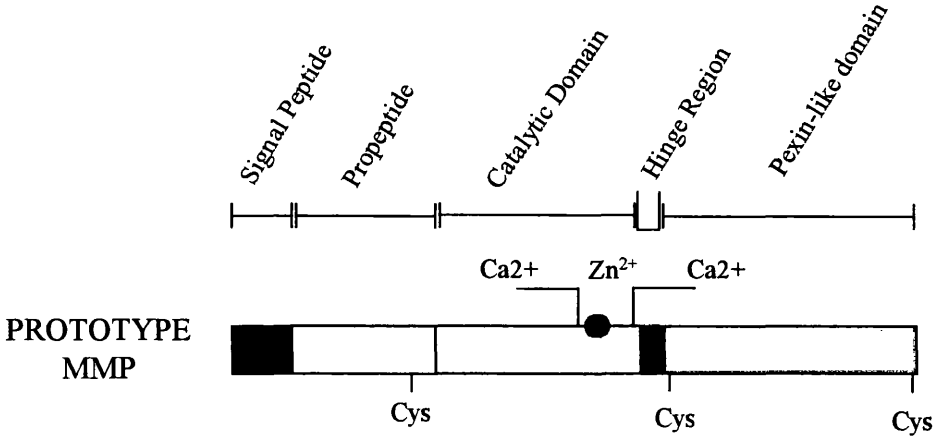


Fig. 1.4 ***Domain structure of a proto-typical MMP.*** (Adapted from [69]).

The MMPs most likely to be involved in TBM turnover are the gelatinases since their primary substrate is collagen IV, a major constituent of the basement membrane [69]. Human PTE are known to express MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [46, 74] and, although some studies describe the expression of stromelysin in rat PTE, there is limited information regarding this MMP in human tubular epithelia [38]. As with all MMPs, the regulation of gelatinases is tightly controlled and occurs at multiple levels including gene transcription, activation of protein products and by binding to the specific

endogenous inhibitors, the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) [69]. Growth factors such as TGF-β, PDGF, FGF, IL-1 and EGF regulate MMP expression [69]. TGF-β can either positively or negatively regulate MMPs but generally increases gelatinase expression [75-77]. In addition to growth factor regulation, gelatinases can also be modulated by the ECM itself [71]. Studies from our laboratory have demonstrated that rat PTE plated on laminin or collagen IV express higher levels of MMP-2 and MMP-9 than cells grown on plastic, with greatest levels observed on a laminin substrate [24]. In these experiments, not only was MMP expression regulated by the matrix but the direction of secretion was also altered; in PTE cultured on laminin apical secretion predominated whereas on other substrates, MMPs were released basally [24].

The TIMPs are a family of inhibitors of which 4 members have been identified to date, TIMP 1-4 [78-80]. TIMP-1 and -2 form 1:1, non-covalent, irreversible bonds with the active forms of all MMPs but they also form tight binding complexes with the latent forms of MMP-9 and MMP-2 respectively [72]. More recently, a third MMP-inhibitor complex has been discovered with the binding of pro-MMP-2 to TIMP-4 [81]. TIMPs can also promote activation of MMPs by formation of ternary complexes; TIMP-2 can activate pro-MMP-2 by MT-MMP1, although the mechanism for this remains unclear [82]. Production of TIMPs is, like that of MMPs controlled by cytokines and growth factors [83]. Currently, only TIMP-1 and -2 have been shown to be expressed by human PTE [84,85], TIMP-3 expression is high in normal mouse kidneys with respect to other organs [79] but to date, the only renal cells which have been reported to produce TIMP-3 are interstitial fibroblasts [86].

Increased TIMP-1 is a common feature of both human progressive renal diseases and animal models of TIF whilst MMP levels are often model- and enzyme-

dependent [5]. In the PAN nephrosis model of tubulo-interstitial fibrosis in rats, immunostaining and *in situ* hybridisation demonstrate TIMP-1 expression along the tubular brush border and within tubular readsorption droplets [49]. Other models describing pathological TIMP-1 induction include obstructive nephropathy [86], protein-overload proteinuria [38] and anti-tubular basement membrane nephritis [87]. Elevated levels of this inhibitor have been demonstrated in human diseases including diabetic nephropathy where renal biopsies show increased TIMP-1 associated with atrophic tubules [85] and in patients with autosomal dominant polycystic kidney disease [88]. Although TIMP-1 levels have been measured in TIF, few studies have investigated TIMP-2 expression; PTE cultured from the Han:Sprague Dawley rat model of polycystic kidney disease have elevated TIMP-2 expression [89] and human PTE treated with glucose in an *in vitro* model of diabetic nephropathy (DN) also have increased expression of this inhibitor [84].

1.2.5 Initiation of fibrosis

Many factors which regulate ECM turnover are thought to be important in tubulo-interstitial disease but relatively little is known about what sets in train the progressive fibrotic process [10]. As mentioned previously, changes in PTE occur early in fibrosis but since many tubulo-interstitial fibroses are glomerular in origin, the essential link between these two compartments is not immediately obvious [66]. Possible mediators of fibrogenesis include cytokines, growth factors, proteolytic enzymes, iron, reactive O_2 metabolites and mechanical injury induced by tubular [3,9,26,43] however two main theories have emerged which may explain the connection between glomerular injury and TIF; the first involves the inappropriate filtration of proteins, lipids and other

macromolecules as a result of increased glomerular permeability [35,42] while the second implicates localised ischaemia resulting from microvascular damage [10,90].

i) Proteinuria

Proteinuria, the appearance of high levels of urinary protein, is a common feature of progressive renal diseases and correlates well with the decline in renal function [35]. The relative importance of protein in the urine has been examined extensively in the rat model of protein-overload where the daily intravenous injection of bovine serum albumin induces tubulo-interstitial inflammation and scarring [38]. Conversely, it has been shown that the restriction of dietary protein, both in patients and in animal models, can retard the progression of disease [57, 91].

High levels of proteins such as transferrin, albumin and lipoprotein in the glomerular filtrate are thought to arise through altered permselectivity of the glomerular capillary barrier, possibly as a result of glomerular hypertension [35,92]. Plasma proteins then pass into the Bowman's space and down into the proximal tubule where injury to the epithelia may occur either through direct toxic effects of the proteins or as the result of resorption of the increased protein load [42]. The high energy levels required to process large amounts of cellular protein stimulates mitochondrial activity which rapidly utilise ATP stores [35]. In addition, lysosomes swell and rupture [66], releasing injurious lysosomal enzymes into the cytoplasm which cause cell death; a theory supported by the presence of lysozyme in the urine of protein-overloaded rats [66,93]. Furthermore, exogenous protein can activate complement in PTE, initiating a cascade of proteolytic reactions which destroy the cells through formation of membrane attack complexes, thereby providing another mechanism of tubular injury [94].

In addition to the toxic effects on cells, the presence of filtered proteins in the tubule may initiate an inflammatory response [95]. *In vitro* investigations have demonstrated that overload of PTE with albumin or IgG induces the expression of chemokines such as MCP-1 and osteopontin [96,97]. *In vivo*, tubular secretion of these molecules could, potentially, attract macrophages into the site of injury and initiate the inflammatory cascade of events which precedes tubulo-interstitial disease.

As well as promoting inflammation, it is likely that aberrant filtration of protein may contribute directly to the fibrotic response. In the protein-overload model there is increased expression of matrix proteins such as collagen IV and laminin in proximal tubules together with elevated levels of the protease inhibitors TIMP-1 and PAI-1, effects which would lead to an overall accumulation of ECM [38]. In addition, *in vitro* studies have shown that human PTE grown on permeable membrane supports and exposed to serum proteins at the apical surface (as would occur in proteinuria *in vivo*), have increased basolateral secretion of fibronectin [98]. It is unclear at present how these effects of protein overload are mediated, one possibility is via induction of pro-fibrogenic cytokines such as TGF-β1 and ET-1 which are both up-regulated in PTE exposed to high protein [38, 99].

ii) Ischaemia

Studies in our laboratory have begun to explore the hypothesis that ischaemia, arising as a result of microvascular damage, may be an essential link between glomerular injury and tubulo-interstitial fibrosis [10,90]. In support of this suggestion, is the observation that progressive obliteration of post-glomerular capillaries correlates with the degree of tubulo-interstitial fibrosis [11]. Since the post-glomerular capillaries emerge to form the vascular network which serves the entire nephron, microvessel

damage would decrease the ability of vessels to deliver O_2, resulting in patchy, local ischaemia within the tubulo-interstitium [11].

The mechanisms which lead to loss of the post-glomerular circulation are not precisely known however one possibility is that efferent damage occurs through glomerular hyperfiltration as a consequence of glomerular capillary hypertension [4,7,11]. Glomerular hyperfiltration results in dilation of the glomerular capillaries which leads to a progressive reduction of post-glomerular blood flow [7]. A clear example of this is in the uniform vasodilation in the remnant kidney model of fibrosis in which all remaining glomeruli are subject to haemodynamic adaptation and hyperfiltration [100]. If endothelial injury ensues and the blood supply to the tubulo-interstitium is compromised, the cells in this tissue compartment become ischaemic [10]. Often systemic hypertension (a common feature of patients with chronically diseased kidneys) is superimposed on this scenario, further damaging the endothelium [96].

Alternatively, the post-glomerular circulation may be compromised by profound obstruction to blood flow through the glomerular capillaries, as is apparent in proliferative glomerulonephritis [1]. Tubulo-interstitial ischaemia arising through vascular stenosis, is thought to occur as a result of local production of vasoconstrictors such as ET-1 and angiotensin converting enzyme (ACE) in response to endothelial damage [92]. This restriction to blood flow may be further exacerbated where vascular sclerosis is a feature of tubulo-interstitial disease, since the diffusion distance for molecular O_2 is greatly increased in these vessels [28].

O_2-deprivation within the tubulo-interstitial compartment would presumably compromise all the cell types, however studies have shown that PTE are particularly O_2-sensitive due to the high metabolic demands conferred by their specialised transport and secretory functions [13,14,101,102]. These demands of metabolism may be even greater

in the presence of proteinuria, since PTE must reabsorb and metabolize the excess load of inappropriately filtered proteins [92]. The combination of high metabolic activity and decreased O_2 delivery therefore makes PTE highly vulnerable to ischaemic injury.

1.2.6 Susceptibility of PTE to hypoxia

Blood flow to the kidney is greater than to any other organ in the body and far exceeds tissue requirements with only 10% of total O_2 delivery utilised by the entire kidney [103,104]. Blood flow within the kidney itself shows striking heterogeneity [104]; the bulk of this supply is directed mostly to the cortex to optimise glomerular filtration and the reabsorption of solutes while delivery to the renal medulla is low to preserve osmotic gradients and enhance urinary concentration [105]. Despite the plentiful supply of O_2 to the kidney, the pO_2 of the TI (3-4%) remains lower than systemic values (10%), rendering the TI relatively hypoxic [106]. Since the medullary blood supply is even lower, this compartment is less well oxygenated with respect to the cortex [105].

Availability of O_2 within the cortex is greater than in the medulla; therefore one would imagine that medullary cells are more vulnerable to hypoxic injury than proximal tubular cells. However, the high metabolic activities of PTE may render them susceptible to hypoxia. In the kidney, 10mins of ischaemia reduces adenosine triphosphate (ATP) levels by 70-90% (hydrolysis of ATP during cellular respiration is utilised to drive energy-requiring cellular reactions [101](*Chapter 2*). Moreover, cortical ATP levels drop much more rapidly than medullary levels [101]. This is thought to be because medullary cells normally utilise glycolytic metabolism for their energy requirements whilst PTE are dependent on oxidative metabolism of fatty acids [101,107]. Furthermore, cells in the medulla can increase their glycolytic rate when oxidative

metabolism is blocked allowing ATP synthesis to continue long after it has ceased in proximal tubular cells [108].

In addition, there is some evidence to suggest that the medulla may have protective mechanisms against ischaemic damage which are absent in PTE [104,105]. Studies have shown that medullary cells rapidly reduce energy-requiring transport functions in during hypoxia, while reduction of work-load by PTE occurs more slowly [104]. Furthermore, the medulla has a higher concentration of heat shock proteins, a family of molecules which protect the cell from stresses such as temperature change, nutrient deprivation and hypoxia, suggesting an additional level of adaptation to ischaemic injury in this region which is absent in PTE cells [105]. It is also possible that although medullary cells are relatively hypoxic with respect to cortex, it is the *change* in pO_2 rather than pO2 levels *per se* which trigger a response to low O_2, thus the effects of hypoxia are greater in PTE than medullary cells. The susceptibility of PTE to ischaemia is therefore attributed to the specialised transport and secretory functions of these cells, the requirement of fatty acid oxidation for ATP generation and the lack of adaptive responses to hypoxic injury.

1.2.7 Ischaemic damage in acute renal failure

There is little information regarding the effects of ischaemia on PTE in the setting of progressive renal disease; however numerous studies have investigated the effects of ischemia-induced acute renal failure (ARF), where the proximal tubule is the primary site of injury [34,101-103]. Although many of these studies are of interest and relevant to a discussion on the cellular response to O_2 deprivation, it must be recognised that generally, *in vivo* models of acute renal failure examine the effects of an acute anoxic (0% O_2) rather than a hypoxic insult, since ischaemia is often achieved via clamping of

the renal artery (which leads to complete cessation of blood flow to the kidney) [109-111]. It is likely that *in vivo*, the severity of ischaemia will vary, ranging from a slight decrease in oxygenation to total anoxia. While *in vivo* and *in vitro* studies of ARF give insight into the effects of O_2 depletion on PTE, it is possible that the results of these studies represent the most extreme effects of O_2 depletion on PTE. It must also be remembered that while chronic renal diseases are progressive and ultimately end in renal failure, in most cases of ARF, tubular regeneration leads to full recovery of renal function [34,101,102]. With these considerations in mind, it is still useful to examine the cellular changes in PTE in response to an brief ischaemic insult as they may be relevant at some level, to ischaemia-induced chronic renal failure.

Depletion of ATP through ischaemia is extremely disruptive to normal cellular and biochemical activity in PTE [101-103] since many of the specialised functions are highly energy-dependent [101]. PTE subjected to ischaemia/reperfusion have impaired protein synthesis, altered membrane transport and loss in cell polarity (discussed further in *Chapter 2*)[101, 109, 112, 113]. If the cellular injury induced by ischaemia is severe, apoptosis or necrosis may ensue and the loss of functional tubules leads to a rapid decline in renal function [101,114,115]. In addition, cellular injury to the remaining tubules may occur when normal oxygenation is resumed due to the formation of reactive oxygen species (ROS) (ischaemia/reperfusion injury) [116-118]. As mentioned previously however, in most cases of ARF, the tubular cells retain the capacity to regenerate and renal function is restored [34].

In considering the potential fibrogenic effects of ischaemic injury, nothing is known regarding the effects of a brief ischaemic episode on ECM metabolism by renal cells but one study of ARF suggests that re-oxygenation after a brief anoxic insult can affect expression of matrix proteins within the kidney; with increased immunostaining for

laminin and fibronectin observed in the PTE-BM after 45mins of re-oxygenation following anoxic injury, presumably as part of a repair process [119].

1.2.8 Gene regulation by hypoxia

Hypoxia is a potent regulator of the expression of a variety of genes [120-122](*Table 1.3*) including those encoding growth factors (such as TGF-β [123], VEGF [124], PDGF [123]), vasoactive molecules (ET-1 [62], inducible nitric oxide synthase [125] and haem oxygenase-1 [126]) and enzymes of cellular metabolism (such as glyceraldehyde-3-phosphate dehydrogenase [127], phosphoglycerate kinase 1 [128] and lactate dehydrogenase A [128]). The regulation of a particular gene by hypoxia is cell type-dependent [123] and most of the reported examples of regulation of gene expression by hypoxia are of increased expression with only limited examples of decreased expression (see *Table 1.3*). Hypoxia can alter gene expression via both transcriptional and post-transcriptional mechanisms [120]. Transcriptional gene regulation by hypoxia was first described for the erythropoietin (EPO) gene [132,144]. EPO is a glycoprotein hormone produced by specialised cells of the kidney and liver [145] that governs the rate of red cell production and hence the O_2-carrying capacity of the blood. In response to hypoxia, transcription of the EPO gene is stimulated and EPO production is induced to increase circulating levels in the plasma by up to a 1000-fold [145]. EPO binds to receptors on erythroid progenitor cells to initiate proliferation and differentiation into mature red blood cells [146]. In this way an O_2-regulated feedback loop links tissue oxygenation to red cell production to increase O_2-carrying capacity and maintain homeostasis [146].

Several hypoxia response elements (HRE) have been identified in the EPO gene [147,148], the predominant one is located within the 3' flanking sequence and contains

Gene	Function	Regulation by hypoxia	Refs.
Positively regulation:			
Aldolase A and C	Glycolysis	↑	[129]
Enolase A	Glycolysis	↑	[130]
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	↑	[127]
Lactate dehydrogenase A	Glycolysis	↑	[128]
Phosphoglycerate kinase I	Glycolysis	↑	[128]
Phosphofructokinase L and C	Glycolysis	↑	[129]
Pyruvate kinase M	Glycolysis	↑	[129]
Glucose transporters 1 and 3	Glucose transport	↑	[131]
Inducible nitric oxide synthase	Nitrix oxide synthesis	↑	[125]
Endothelin-1	Vasomotor regulation	↑	[62]
Erythropoietin	Erythropoiesis	↑	[132]
Haem oxygenase-1	Haem metabolism	↑	[126]
Transferrin	Iron transport	↑	[133]
Tyrosine hydroxylase	Catecholamine synthesis	↑	[134]
Adenylate kinase-3	Phosphate metabolism	↑	[135]
IGF-II	Growth factor	↑	[136]
TGF-β	Growth factor	↑	[123]
TNF-α	Growth factor	↑	[137]
PDGF-BB	Growth factor	↑	[123]
Placental growth factor	Growth factor	↑	[123]
VEGF	Growth factor	↑	[124]
IL-1, -6, -8	Interleukins	↑	[138-40]
Negative regulation:			
Glucose transporter 2	Glucose transport	↓	[131]
Phosphoenolpyruvate carboxykinase	Gluconeogenesis	↓	[141]
Endothelial nitric oxide synthase	Nitrix oxide synthesis	↓	[142]
Angiopoietin	Angiogenesis	↓	[143]

Table 1.3 ***Hypoxia-inducible genes and their functions.***

the core consensus binding region: 5'-CGTG- 3' which provides a binding site for the specific transcription factor hypoxia-inducible factor-1 (HIF-1) [149]. Since the discovery of this *cis*-acting HRE, many other hypoxia-inducible genes have been shown to contain this same consensus sequence which may be located in the 3' flanking region, 5' flanking region or within the gene itself [150-151]. The sequences flanking this core motif vary between genes however an adenosine residue often precedes the obligatory core sequence (5'-(A)CGTG-3') [151]. It has also been observed that adenosine rarely appears immediately after the core sequence (5'-CGTG(A)-3'); the significance of the bases adjacent to the HIF-1 core motif is yet to be determined [151].

HIF-1 is a DNA-binding heterodimer comprised of 2 basic-helix-loop-helix (bHLH) proteins, HIF-1α and HIF-β [152]. The α sub-unit is unique to HIF-1 and is precisely regulated by cellular O_2 concentration such that levels of HIF-1α protein and HIF-1 DNA binding activity increase exponentially as O_2 concentration decreases [153]. In contrast to the specificity of HIF-1α, the β sub-unit was first identified as the aryl hydrocarbon nuclear translocator (ARNT) and is not regulated by hypoxia [152]. HIF-1β can heterodimerize with other bHLH proteins including the aryl hydrocarbon receptor (ARH) and Single-minded-2 [152]. The recent finding that HIF-1β can also recognise the HIF-1 binding sequence when dimerized with HIF-2α (also known as EPAS-1/HLF/HRF/MOP2) suggests that HIF-1 is part of a larger family of hypoxia-inducible transcription factors [154,155]. In most cases, HIF-1 cannot induce transcription independently and requires accessory factors to activate the gene, such as HNF-4 and ATF-1/CREB-1 [156,157]. A number of other transcription factors are also induced by hypoxia including NF-κB and AP-1, although it is currently unclear whether these act in association with HIF-1 [158,159] (discussed in more detail in *Chapter 5*).

In addition to transcriptional regulation, hypoxia can modulate mRNA expression, either positively or negatively by altering the stability of specific mRNAs [120]. For example, hypoxia-induced VEGF gene transcription is regulated by the presence of a HIF-1 site within the 5'flanking sequence of the gene [160], however the presence of an mRNA stabilising element in the 3' untranslated region provides a further level of regulation [161]. Stability of VEGF mRNA is conferred by binding of the nuclear protein hnRNPL (heterogeneous nuclear ribonucleoprotein L) to the mRNA stabilising element which prevents pre-mRNA processing and splicing, thus increasing steady-state VEGF mRNA levels [162].

1.2.9 Effect of hypoxia on ECM metabolism

Although hypoxia is known to regulate a wide variety of genes, currently there is limited information on the effects of O_2 depletion on genes involved in ECM metabolism in any cell type; one preliminary report (in abstract form) suggests that in mesangial cells, hypoxia stimulates collagen gene expression [163]. In this case increased collagen I mRNA levels were attributed to hypoxia-induced TGF-β1, although this was not proven [163]. Hypoxic-induction of matrix proteins has been described in 2 non-renal systems; studies on dermal fibroblasts describe a hypoxia-induced upregulation of collagen α1(I) mRNA [164] and models of hypoxia-induced neonatal pulmonary hypertension have shown an up-regulation of tropoelastin, fibronectin and collagen I mRNA in the pulmonary artery wall [165]; however in none of these examples have the mechanisms underlying matrix gene induction in response to hypoxia been identified. Similarly, the effect of hypoxia on aspects of matrix degradation have not previously been investigated in the kidney and there is only limited information from non-renal cells. One *in vitro* study demonstrates that MMP-9 secretion is stimulated by hypoxia in

human keratinocytes [166] while investigation of the plasminogen pathway in response to hypoxia has shown that PAI-1 mRNA expression is increased in human trophoblasts [167] and murine macrophages [168].

As mentioned previously, one feature of TIF is thickening of the TBM as a result of increased synthesis and decreased degradation [5]. In addition to qualitative changes the TBM also has altered composition, with the appearance of 'interstitial' matrix proteins such as collagen I [50,51]. The mechanisms underlying these changes remain obscure, however one possibility is that hypoxia arising from microvascular injury *in vivo* may provide a stimulus for qualitative and quantitative changes in the TBM. The effects of hypoxia on PTE ECM metabolism have not been studied previously. Therefore the aim of this project was to examine the hypothesis that hypoxia can elicit changes in PTE ECM synthesis and degradation, acting as a pro-fibrogenic stimulus for this cell-type.

1.3 Aims

- To define an *in vitro* model of hypoxia which induces a sub-lethal injury in PTE.
- To establish whether hypoxia affects ECM synthesis and/or degradation.
- To determine the mechanism(s) of hypoxia-induced changes in ECM metabolism.

CHAPTER 2

Effects of hypoxia on PTE growth and metabolism

2.1 Background

PTE are highly metabolic cells with high energy requirements for specialised secretory and transport functions [13,101,102]. *In vivo*, O_2 consumption provides the major source of energy, through oxidative phosphorylation which efficiently generates the high levels of ATP required to meet PTE metabolic demands [101,107]. During an ischaemic insult however, O_2 is depleted and cells rapidly become dependent on anaerobic respiration for energy [102]. Since the ATP yield produced by glycolysis is small compared to that generated by aerobic respiration [169], the energy produced under hypoxic conditions is insufficient to meet PTE metabolic demands [101]. Consequently, PTE undergo rapid changes; cells have altered membrane transport, loss of cell polarity and impaired protein synthesis [101,109,112,113]. If the ischaemic injury is severe, (eg. sustained anoxia) cell death may result (see *Chapter 1: 1.6*) [101,114,115].

The marked effects of ischaemia on PTE are partly due to the high metabolic requirements of these cells but there is also evidence that PTE lack adaptive mechanisms for hypoxia tolerance [104,105]. A comparative study investigating the effects of hypoxia on PTE compared to pulmonary arterial endothelial cells (PAEC) *in vitro*, demonstrated that while PAEC are relatively tolerant to O_2 depletion, PTE are extremely hypoxia-sensitive [14]. Hypoxia-tolerance in specific cell types is usually attributed to one or more of the following features: large stores of fermentable fuel, high tolerance of metabolic acidosis (arising from glycolysis), metabolic rate depression and improved

handling of cellular calcium and reduced membrane permeability [14,101,102]. Recent studies have indicated that of these mechanisms for hypoxia-tolerance, the most effective are metabolic arrest and the ability to preserve plasma membrane integrity [169,170].

Under normoxic conditions, the high energy phosphate ATP, is hydrolysed to ADP and AMP, to release free energy [171]. During hypoxia however, these phosphates are further degraded to the nucleosides adenosine and inosine and the base hypoxanthine. [101,102] (*Fig. 2.1*).

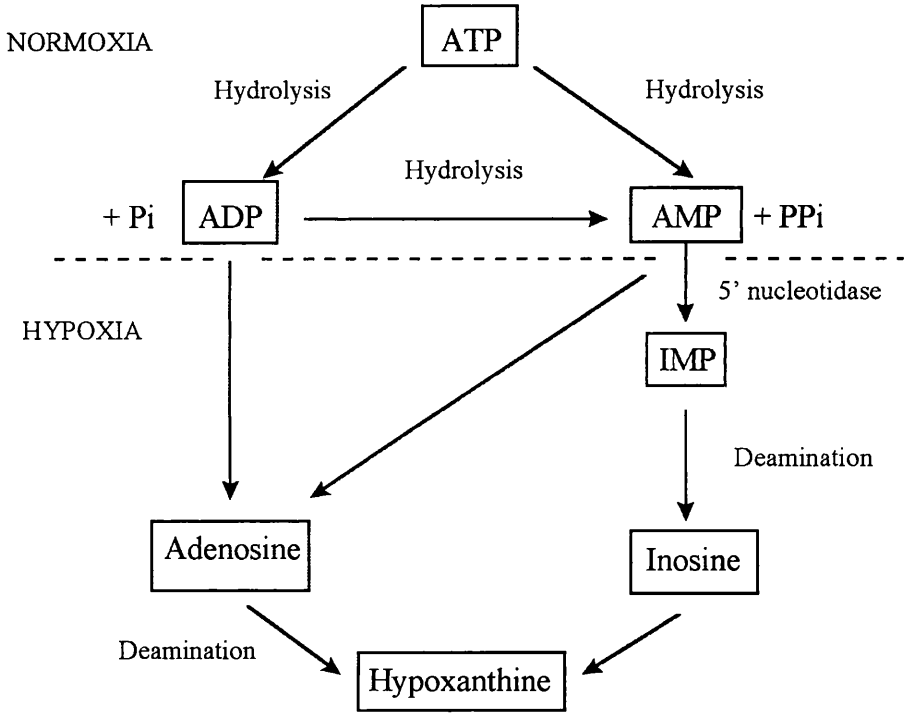


Fig. 2.1 ***ATP metabolism in normoxia and hypoxia*** [101,102].

Hypoxia-tolerant cells such as PAEC have the ability to maintain high levels of ATP during O_2 depletion, by maintaining/increasing the rate of glycolysis and decreasing the rate of ATP hydrolysis [14]. In cells adapted to withstand hypoxia, ATP turnover is coupled to ATP synthesis, ultimately leading to metabolic arrest during periods of hypoxia [14]. As a further protective measure, 'channel arrest' occurs in some cell

types, for example, it has been reported that in an attempt to conserve energy, Na+/K+ATPase activity is suppressed in hepatocytes exposed to hypoxia [170]. Hypoxia-sensitive cells such as PTE do not undergo metabolic arrest and consequently ATP levels rapidly decline during hypoxia, resulting in cellular injury [14]. In addition, the rapid increase in membrane permeability results in the inappropriate passage of solutes and ions across the cell membrane, altering cell volume and depriving the cell of important metabolic precursors [101]. It has been demonstrated that in PTE exposed to hypoxia, the cellular nucleoside pool is rapidly depleted [14]. While the nucleotides ATP, ADP, AMP and IMP are relatively impermeable to cells; the nucleosides adenosine, inosine and hypoxanthine, can leak across the cell membrane [101,102]. If, as in PTE, membrane permeability is increased, the loss of nucleosides prevents re-synthesis of ATP molecules from existing pre-cursors. Presumably valuable energy must then be utilised for *de novo* synthesis of nucleosides.

As mentioned, the effects of ischaemia on PTE can be severe, however the damage incurred to cells when oxygenation is restored is often greater than the ischaemic injury itself [116,118]. Ischaemia/reperfusion injury has been extensively studied in the context of ARF where the injury resulting from a transient ischaemic insult is greatly exacerbated once normal blood flow is restored [101,103]. This effect is due to an increase in free radical ROS production by PTE on re-oxygenation. [116-118]. Production of these highly reactive free radicals and ROS on re-oxygenation induces lipid peroxidation which has a number of deleterious effects: plasma and subcellular membrane permeability is increased, enzymatic processes and ion pumps are impaired and DNA is damaged [101]. In addition, direct oxidation of membrane protein occurs, affecting critical proteins such as Na^+/K^+ ATPase and the Ca^{2+} ATPase [172] with consequences on cellular ion transport functions.

The effects of O_2 depletion on PTE have been extensively described within the context of ARF [101,103,173] but as mentioned in *Chapter 1*, it must be recognised that generally, *in vivo* models of ARF examine the effects of an anoxic (0% O_2) rather than a hypoxic insult [109-111]. However it is useful to consider the data obtained from ARF studies as it is likely that these represent the most extreme effects of O_2 deprivation on PTE.

In vivo, PTE subjected to ischaemia/reperfusion injury undergo a number of biochemical changes, some of which are reflected in cellular morphology [102,174] (*Fig.2.2*). Data from *in vivo* models describe altered cell size and shape; cells may become swollen due to increased membrane permeability or cytoskeletal disruption [101,102]. In addition, the plasma membrane becomes ruffled due to loss of desmosomal contacts [34,173]. Membrane blebbing becomes apparent with internalisation or shedding of microvilli and gradual loss of membrane selectivity [101,173]. This leaky membrane then allows the inappropriate entry of ions such as calcium which accumulate to toxic levels in the mitochondria [174].

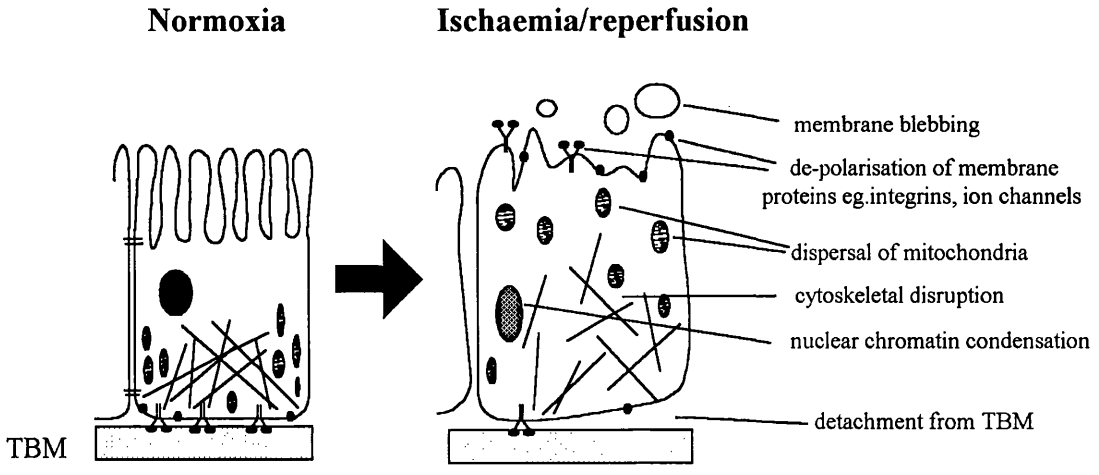


Fig. 2.2 ***Schematic diagram illustrating the effects of ischaemia/re-perfusion on PTE in vivo [99,170].***

One important feature of ischaemic injury is loss of cell polarity resulting from disruption of the actin cytoskeleton and the opening of tight junctions [175,176]. Many epithelial cell transport functions are dependent on the appropriate localisation of proteins to either the apical or basolateral membranes [20]; in ischaemia, however, cell polarity is lost, with, for example, proteins such as the Na^+-K^+-ATPase located in the apical domain and leading to the inefficient reabsorption of Na^+ ions [113]. In addition to the severe impact on transport function, loss of polarity also leads to sloughing of viable cells [102] as the basolateral integrin ECM receptors which mediate cell-matrix attachment, are re-located to the apical surface, causing cells to detach from the ECM [177]. For example, in the rat kidney, the β1 integrin sub-unit is localised to the basolateral membrane of PTE but following an ischaemic insult this sub-unit is directed to the apical surface [178], resulting in cell detachment. Further injury ensues when desquamated cells aggregate, or attach to cells remaining *in situ*, via integrin receptors recognizing the arginine-glycine-aspartic acid (RGD) sequence presented by matrix proteins [178,179]. This accumulation of sloughed cells within the tubular lumen, leads to formation of the obstructive casts which are observed in acute renal failure [177]. *In vivo* studies have demonstrated that apically expressed integrins can be inhibited by the administration of RGD containing peptides and may therefore be of therapeutic use in the amelioration of ischaemic acute renal failure [180].

If the injury imposed by anoxia/re-oxygenation is severe, cell death ensues [101-103]. Until recently, cell death arising from ischaemia/reperfusion injury was thought to be only by necrosis, but some investigators have reported apoptosis (or programmed cell death) as an early form of cell death in ARF [114,115,181]. Cells undergoing apoptosis are characterised by endonuclease activation resulting in degradation of genomic DNA, chromatin condensation at the nuclear periphery, compaction of the cytosol and

breakdown of epithelial desmosomal attachments [101]. Apoptotic cells can be recognised in culture as spherical bodies, formed from blebs of plasma membrane containing condensed cytosol and nuclear fragments [101].

Apoptosis has been demonstrated in primary cultures of rat PTE exposed to free radicals (to mimic ischaemia/reperfusion injury) [181] however the evidence of apoptosis from *in vivo* models of ARF is contentious. In an animal model of ARF induced by renal artery clamping, Schumer et al describe apoptosis, as measured by DNA laddering, continuing up to 48hrs after 5mins of ischaemia, with no associated necrosis [114]. However studies by Zager et al failed to demonstrate any DNA laddering in a similar model using the same technique [115]. DNA damage was only observed on application of the more sensitive terminal deoxynucleotidyl transferase (tdt) technique, whereby formalin-fixed tissues are incubated with tdt and biotinylated deoxyuridine which are incorporated into exposed DNA breaks. DNA damage detected in this fashion appeared post-ischaemia concomitant with the morphologic appearance of necrosis not apoptosis [115], demonstrating that further studies are required in order to assess the role of apoptosis in renal ischaemia/reperfusion injury.

Despite the overt tubular cell death which occurs as a result of ischaemia/reperfusion, PTE, which in the normal adult kidney, are a relatively quiescent cell population, have a remarkable capacity for regeneration and as mentioned previously (*Chapter 1:1.7*), full recovery occurs in most cases of ARF [34]. The process of tubular regeneration in surviving PTE is poorly understood but it is likely that autocrine or paracrine release of growth factors and cytokines in response to injury may induce cells to leave the G_0 phase of the cell cycle, initiating DNA synthesis so that cells undergo mitosis [34]. Previous studies in our laboratory have shown that human PTE exposed to 1%O_2 for 48hrs are stimulated to proliferate [182] and that an autocrine growth loop

exists for endothelin-1 released by PTE in response to hypoxia [62]. Increased PTE proliferation in response to an ischaemic insult *in vivo* would presumably ensure re-population of the proximal tubule after ARF, restoring normal tubular activity.

As mentioned in *Chapter 1*, the overall aim of this project was to examine the role of chronic hypoxia as a pro-fibrogenic stimulus in PTE. As a first step, it was necessary to establish an appropriate *in vitro* system for exposure of cells to hypoxia. Previous studies in our laboratory have investigated the effect of hypoxia on PTE proliferation, however the effects of hypoxia on human PTE protein production and metabolism have not previously been described in an *in vitro* system.

2.2 Aims

- To develop an appropriate *in vitro* model to examine the effects of 'chronic' hypoxia on primary cultures of human PTE.
- To establish that the hypoxia conditions induce metabolic changes but do not result in cell death.

2.3. Methods

2.3.1 Culture of human PTE

Human PTE were isolated and cultured from explant tissue using a standard protocol. PTE used in this study were derived from the histologically normal pole of human kidney removed for carcinoma. Tissue was minced, passed through a 180μm sieve (BDH-Merck Ltd., Lutterworth, Leics, UK) to remove the glomeruli, plated on rat tail collagen I-coated or uncoated tissue culture plastic (Greiner Labs, Dursley, Glos, UK) and grown in RPMI 1640 medium [183] supplemented with 10% foetal calf serum (FCS), adenosine,

guanosine, uridine, cytosine (12.5mg/ml each), transferrin (5μg/ml), insulin (5μg/ml), dexamethasone ($5x10^{-8}$M), 1% antibiotic/antimycotic (penicillin (100U/ml), streptomycin (100μg/ml)), amphotericin B (25ng/ml)), HEPES (10mM) (all reagents from Sigma Chemical Company, Poole, Dorset, UK except RPMI 1640 and FCS which were purchased from Gibco-BRL, Paisley, Scotland, UK). Cellular outgrowths from the tissue fragments appeared 3-4 days after plating. Cells had a cobblestone morphology characteristic of epithelial cells (*Fig. 2.3*).

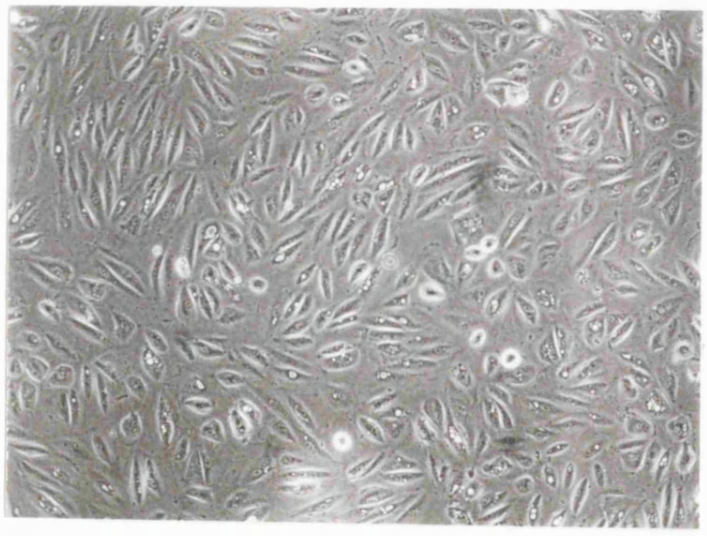


Fig 2.3 ***Photomicrograph of human proximal tubular cells (PTE) at passage 2.*** Magnification: x100.

The medium was changed every 3-4 days and cells were passaged at confluence using trypsin-EDTA (trypsin 0.5mg/ml, EDTA 0.2mg/ml; Gibco-BRL). Confluent cells were made quiescent by 24hrs incubation in serum-free RPMI 1640 medium containing nucleosides, HEPES, transferrin and antibiotic/antimycotic (quiescence medium).

2.3.2. Immunocytochemistry

For immunocytochemical characterisation, PTE, were grown to semi-confluence (approximately 80% cover) in 8-well chamber slides (Gibco-BRL) and made quiescent. For intracellular proteins, cells were fixed with ice-cold methanol at -20°C. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in methanol for 30 mins at room temperature. Cells were washed twice in phosphate buffered saline (PBS) with Tween-20 (0.02%; Sigma) for 15mins and blocked in 1.5% serum derived from the species in which secondary antibody was raised (Vectastain *Elite* ABC Kit, Vector Labs, Bretton, Peterborough, UK) for 20mins at room temperature. Primary antibodies were diluted in 2% bovine serum albumin (BSA) in PBS: monoclonal anti-pan cytokeratin (CAM 5.2, 1:100 dilution; Roche Molecular Biochemicals, Lewes, Sussex, UK), monoclonal anti-vimentin (clone Vim 3B4, 1:250; Roche Molecular Biochemicals), monoclonal anti-α-smooth muscle actin (1:1000; Sigma), monoclonal anti-desmin (1:1000; Sigma). Cells were incubated with primary antibodies for 45mins at room temperature, washed 3 times with PBS for 10mins each and incubated with biotinylated secondary antibodies (Vectastain *Elite* ABC Kit, Vector Labs.) diluted in PBS (containing 1.5% blocking serum) for 30mins at room temperature followed by avidin-biotin complex (ABC; Vectastain *Elite* ABC Kit, Vector Labs.) for 45mins at room temperature. Positive reactivity was visualised using the chromogenic substrate aminoethylcarbazole (AEC-Substrate Kit, Vector Labs.) giving a red-brown precipitate. Cells were counterstained with Harris' haematoxylin (Sigma) for 30secs at room temperature, rinsed in running water, mounted in Aqua-Polymount (Polysciences Ltd, Molton Park, Northants, UK) and examined on a Zeiss Axiophot microscope (Carl Zeiss (Oberkochen) Ltd, Welwyn Garden City, Herts, UK).

By immunocytochemistry, cells between passage 1-6 were positive for cytokeratin (an epithelial cytoskeletal protein) but negative for vimentin (a marker of

cells of mesodermal origin), α-smooth muscle actin and desmin (markers characterististic of myofibroblasts and mesangial cells). These data suggest that the cellular outgrowth consisted predominantly of tubular epithelial cells. For experiments, cells were used up to passage 6 since immunocytochemistry of cells between passage 7-9 showed some positive staining for vimentin, suggesting possible de-differentiation with repeated passaging *in vitro*.

2.3.3. Assay for alkaline phosphatase activity in PTE

Alkaline phosphatase is an enzyme localised exclusively within the brush border of proximal tubular epithelial cells. To examine alkaline phosphatase activity in PTE, confluent cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS (containing 0.9mM Ca^{2+} and 0.5mM Mg^{2+}) for 5mins at room temperature, washed twice in PBS for 10mins and stored at 4°C prior to assay. Fixed cells were incubated in 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 150μg/ml; Roche Molecular Biochemicals) and 4-nitro blue tetrazolium chloride (NBT, 300μg/ml; Roche Molecular Biochemicals) in 100mM Tris pH 9.5, 100mM NaCl, 5mM $MgCl_2$ (Sigma) at room temperature in the dark overnight or until the appearance of a purple colour. Cells were positive for alkaline phosphatase (>90%) suggesting that cultures were composed predominantly of PTE [24].

2.3.4 Hypoxic conditions

To examine the effects of hypoxia, cells were made quiescent for 24hrs as described in *2.2.1.* Fresh quiescence medium was added and cells incubated in 1% O_2/5% CO_2/94 %N_2 (pre-mixed special gas; British Oxygen Company Ltd., Luton, Beds, UK) in a Billups Rothenberg Chamber™ (ICN-Flow, High Wycombe, Bucks, UK) for 24hrs.

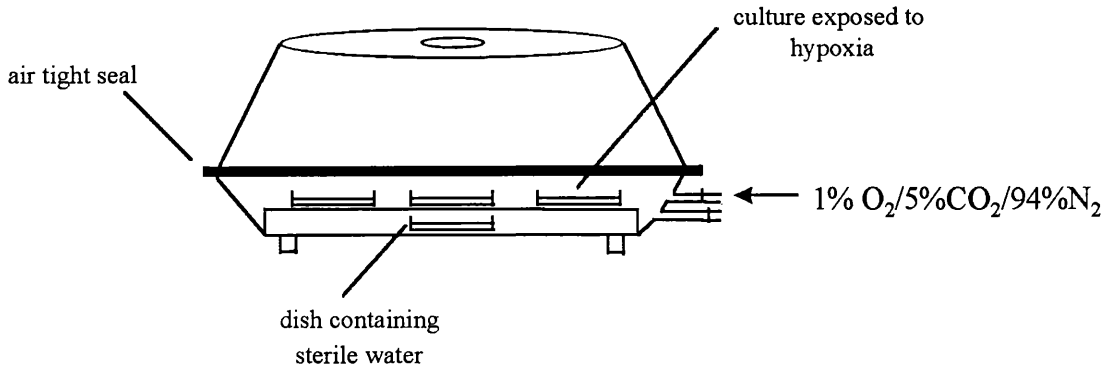


Fig. 2.4 ***Diagram of Billups-Rothenberg™ hypoxia chamber.*** Cultures were placed in the chamber and gassed for 20mins with 1% O_2. A dish of sterile water was placed at the base to humidify the chamber. After gassing, the chamber was sealed and placed at 37°C for 24hrs. At the end of the incubation time, the gas in the chamber was analysed to ensure that the chamber had remained air-tight.

For time-course experiments, cells were exposed to hypoxia for 1, 2, 4, 8 and 24hrs. The O_2 concentration inside the chamber was confirmed at the end of the hypoxic period using a Radiometer ABL4 (Radiometer, Copenhagen, Denmark). For re-oxygenation experiments, at the end of the hypoxic period the medium was replaced with fresh quiescence medium and the cells transferred to normoxia (21%O_2) for 24hrs (referred to as the 're-oxygenation' period). Conditioned medium (CM) was collected at the end of hypoxia and at the end of the re-oxygenation period and cells harvested for RNA extraction (*2.3.8*). CM were clarified by centrifugation at 2,000xg for 15mins at 4°C and stored at -80°C prior to use. Total protein content of CM was measured using a modified Bradford Assay [24] with 0.06% Coomassie Brilliant Blue G-250 in 0.3% perchloric acid (both reagents from BDH-Merck) and read at 595nm using a DU-64 Spectrophotometer with the Soft-Pac™ Protein Assay Module (Beckman Instruments Ltd., High Wycombe, Bucks, UK). Protein concentration was determined by comparison to BSA standards (0-20μg/ml) prepared in quiescence medium.

2.3.5 Measurement of Cell Number and Cell Viability

Cell number was counted using a haemocytometer (Marathon Laboratory Supplies, London, UK). Cell viability was measured using both Trypan Blue exclusion and LDH release. For the Trypan Blue assay, aliquots of cell suspension were mixed (1:1) with Trypan Blue (Sigma). Trypan Blue permeates the membranes of dead cells, giving these a blue coloration but is actively excluded by the functioning cell membrane of living cells which do not undergo colour change. The number of dead cells (stained blue) were calculated as a percentage of the total cell number. Lactate dehydrogenase (LDH) release (as a percentage of total LDH) was measured using an LDH Assay Kit (Sigma) according to the manufacturer's instructions.

2.3.6. Measurement of Cell Proliferation

Confluent, quiescent PTE were incubated with 2μCi/ml ^{3}H-Thymidine (^{3}H-TdR; 26Ci/mmol; Amersham International plc, Little Chalfont, Bucks, UK) 1ml/well of a 24-well plate for 2hrs prior to harvest. Cells were washed with PBS and cellular protein and DNA precipitated with ice-cold trichloro-acetic acid TCA (10%) overnight at 4°C. TCA was removed, and the precipitate solubilized with 0.5M NaOH. Aliquots were added to Ecoscint-A scintillation fluid (National Diagnostics, Hessle, Hull, UK) and radioactivity counted in a Packard 2000CA Tri-Carb Liquid Scintillation Analyser (Canberra Packard, Pangbourne, Berks, UK). Background counts were subtracted from sample values and the data expressed as disintegrations per minute (dpm) per well.

2.3.7 Cellular protein synthesis

For measurement of cell protein synthesis, cells were labelled for 24hrs with 5μCi/ml ^{3}H-Phenylalanine (26Ci/mmol; Amersham) added at the beginning of 24hrs hypoxia or at the

start of 24hrs re-oxygenation. Radioactivity was measured in aliquots of HPLC hydrolysate (100µl) (*Chapter 3: 3.2.1*) and counted in a Packard 2000CA Tri-Carb Liquid Scintillation Analyser. Data are presented as dpm/well.

2.3.8 RNA Extraction

Total cellular RNA was extracted using TRIZOL^R Reagent (Gibco-BRL) according to the manufacturer's instructions. Briefly, cells were lysed with TRIZOL^R Reagent (1ml/10cm dish) directly in the tissue culture dishes. Lysates were transferred to microfuge tubes, incubated for 5mins at room temperature and chloroform added (0.2ml/ml of TRIZOL^R Reagent). Samples were shaken vigorously and incubated for 5mins at room temperature prior to centrifugation at 12,000xg for 15mins at 4°C. The upper aqueous phase was collected and isopropyl alcohol added (0.5ml/ml TRIZOL^R Reagent used for the initial lysis). Samples were vortexed, incubated for 20mins at room temperature and then centrifuged at 12,000xg for 10mins at 4°C. The supernatant was discarded and the RNA pellet washed once with 75% ethanol (BDH-Merck) prepared in diethylpyrocarbonate (DEPC)-treated RNAse-free water (0.02% DEPC, Sigma; the solution was allowed to stand at room temperature for a minimum of 45mins prior to autoclaving). Samples were vortexed and centrifuged at 7,500xg for 5mins at 4°C. The RNA pellet was air-dried and resuspended in 5-20µl DEPC-H_2O water and the concentration of RNA measured by absorbance at 260nm using a DU-64 Spectrophotometer with the Soft-Pac™ Nucleic Acid module (Beckman Instruments Ltd.). RNA concentration was calculated from absorbance values where A_{260} 1=40µg/ml of RNA. Absorbance was also measured at 280nm to give an indication of purity; samples were only used where the 260/280nm ratio was between 1.7-2.0. Samples were stored at -80°C prior to use.

2.3.9 Northern Blot Analysis

For Northern blot analysis, 5-20μg of total RNA (equal concentrations of RNA loaded/lane) and RNA standards (0.24-9.5kb, 1μg/μl, Gibco-BRL) were electrophoresed on a 1% agarose (Sigma) gel, containing 1.85% formaldehyde with 1X MOPS pH 7.0 (20mM MOPS (3-[N-morpholino]propanesulfonic acid), 5mM sodium acetate, 1mM EDTA) containing 3.7% formaldehyde as the running buffer. on each gel. Ethidium bromide-stained gels were photographed under UV illumination to check the integrity of RNA (ratio of 28S:18S ribosomal bands should be approximately 2:1) and for normalisation of RNA loading by densitometry. RNA was capillary-blotted to nitrocellulose membrane (0.2μm pore; Schleicher & Schuell, Dassel, Germany) for 16-18hrs at ambient temperature with 1M ammonium acetate as the transfer buffer and blots baked under vacuum at 80-100°C for 2hrs. Membranes were pre-hybridised overnight at 65°C in 4XSSC (20XSSC: 3M NaCl, 0.3mM Na_3citrate.$2H_2O$, pH 7.0), 5% Denhardt's solution (8mg/ml polyvinylpyrrolidone (PVP), 8mg/ml BSA, 8mg/ml Ficoll; Sigma) 0.5% SDS, 5mM Tris pH7.5, 1mM EDTA pH7.5 in a Techne Hybridisation Oven (Techne Ltd., Duxford, Cambs, UK). cDNA for GAPDH (American Type Culture Collection (ATCC); Rockville, Maryland, USA) was labelled with α^{32}P-dCTP (3000Ci/mmol; Amersham) using a Random Primed DNA Labelling Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Blots were hybridised overnight at 65°C in pre-hybridisation solution containing 50μg/ml pA^+-RNA (Roche Molecular Biochemicals), 100μg/ml sheared, denatured salmon sperm DNA (Sigma) and 10^7 dpm α^{32}P-dCTP-labelled cDNA probe. Blots were washed 4 times, 20mins each, in 4XSSC, 0.5% SDS at 50°C with gentle agitation and exposed to autoradiography film (Kodak XAR; KJP, London, UK) at -80°C with intensifying screens for 1-6 days. Photographs of ethidium bromide-stained gels and autoradiograms were scanned and quantified by densitometry (3D ImageMaster Software; Pharmacia, St Albans, Herts, UK). Signals were corrected for

variations in RNA loading by comparison to densitometry values of ethidium bromide-staining of rRNA. Data are expressed as fold change in mRNA level in PTE exposed to hypoxia, calculated from arbitrary densitometry units with respect to the relevant normoxic controls (assigned an arbitrary value of 1).

2.3.10 Statistical analysis

Data were analysed using Student's T-Test (paired or unpaired, as appropriate). Values are held to be significantly different when the probability of such differences arising, assuming the null hypothesis to be true, are less than 5% ($*P<0.05$). Where mean values are calculated, standard errors of the mean are also given.

2.4 Results

2.4.1. Cell viability

PTE were examined after 24hrs hypoxia ($1\%O_2$) and 24hrs re-oxygenation ($21\%O_2$). Hypoxia had no marked qualitative effect on cell size or morphology compared to control cultures; cells retained their characteristic cobblestone appearance and there was no evidence of altered cell-cell contact or membrane blebbing. There was no quantitative difference in cell number between control and hypoxia cultures or in the degree of cell death as assessed by the number of floating cells per dish. Cell counts confirmed that there was no significant difference in the number of control and hypoxic PTE (*Table 2.1*). Hypoxia had no effect on cell viability as measured by Trypan Blue-exclusion and measurement of LDH released into the media (calculated as a percentage of total LDH) (*Table 2.1*).

	Normoxia (21% O_2)	Hypoxia (1%O_2)
Cell number /10cm dish	$3.27 \pm 0.35 \times 10^6$	$3.30 \pm 0.46 \times 10^6$
Cell viability:		
a. Trypan blue (stained cells/10cm dish)	$1.55 \pm 0.10 \times 10^4$	$1.41 \pm 0.12 \times 10^4$
b. LDH release (% of total LDH released)	44.9 ± 2.2 %	45.5 ± 0.8 %

Table 2.1 *Effect of hypoxia on cell viability of PTE*

These data suggest that exposure of PTE to 1% O_2 for 24hrs does not cause overt cellular injury or cell death. However, it was observed that attachment to the substrate appeared to be greater in cells exposed to hypoxia, with treated cells requiring more prolonged trypsinisation for detachment.

2.4.2. Cell proliferation

The observation that hypoxia had no effect on cell number (*2.4.1*), suggested that cellular proliferation of PTE is not affected by this degree of O_2 deprivation. Measurement of cell proliferation (incorporation of ^{3}H-TdR) after 24hrs hypoxia and 24hrs re-oxygenation also showed there was no significant difference in ^{3}H-TdR incorporation after hypoxia or re-oxygenation with respect to the relevant controls (*Fig.2.5*).

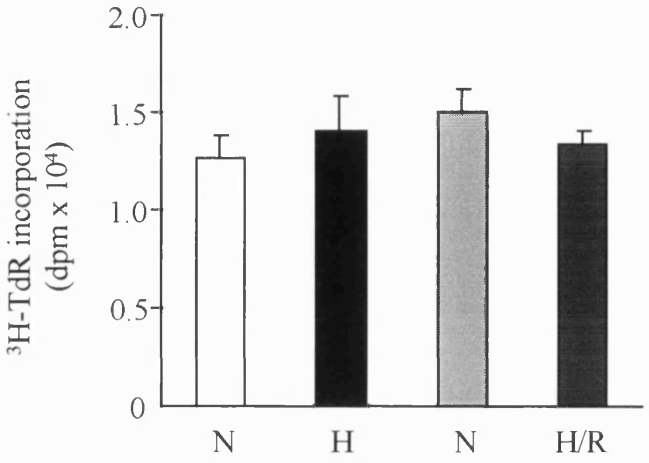


Fig.2.5 ***^{3}H-TdR incorporation of PTE exposed to 24hrs of hypoxia and 24hrs re-oxygenation.*** Confluent, quiescent cells were labelled with 2μCi/ml of ^{3}H-TdR for 2hrs prior to harvest, after 24hrs normoxia (**N**), 24hrs hypoxia (**H**), 48hrs normoxia (**N**) and 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). ^{3}H-TdR incorporation is expressed dpm/well. Data are the mean (±SEM) of 3 experiments with n=4 wells in each sample.

2.4.3. Protein production

As mentioned earlier (*2.1*), hypoxia can alter protein production in other cell types. To assess whether the hypoxia conditions used in the present study had any effect on protein synthesis by PTE, synthesis was measured by the incorporation of ^{3}H-phenylalanine into protein.

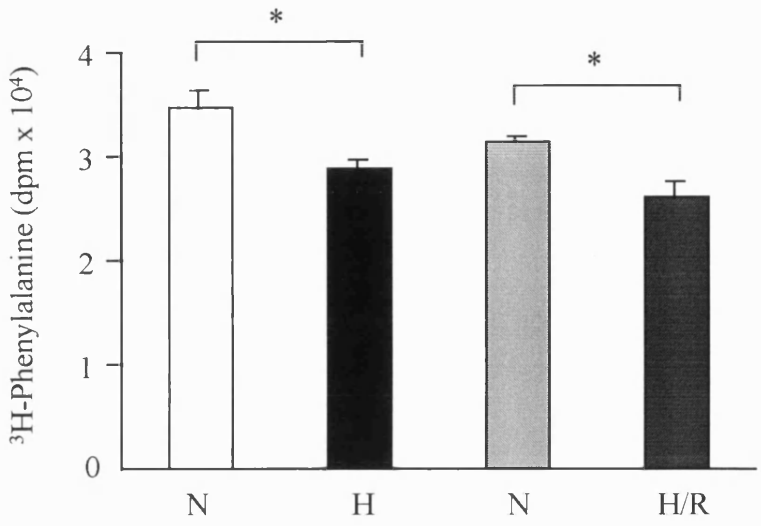


Fig. 2.6 ***^{3}H-Phenylalanine incorporation of PTE exposed to 24hrs hypoxia or 24hrs re-oxygenation.*** PTE used were labelled with 5μCi/ml ^{3}H-phenylalanine:24hrs normoxia, 21% O_2 (**N**); 24hrs hypoxia, 1%O_2 (**H**); 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). Data are the mean of 2 experiments with n=6 wells in each sample, *$P< 0.05$ by unpaired Student's T-Test

As shown in *Fig. 2.6*, protein synthesis was decreased after 24hrs hypoxia and remained suppressed during re-oxygenation.

2.4.4 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression

To assess the effects of hypoxia on metabolic activity of PTE, GAPDH mRNA levels were measured. GAPDH is an important component of the glycolytic pathway, involved in the conversion of glyceraldehyde 3-phosphate to pyruvate and the generation of ATP [171]. GAPDH gene expression was induced by 24hrs hypoxia and remained high in the re-oxygenation period (*Fig. 2.7*).

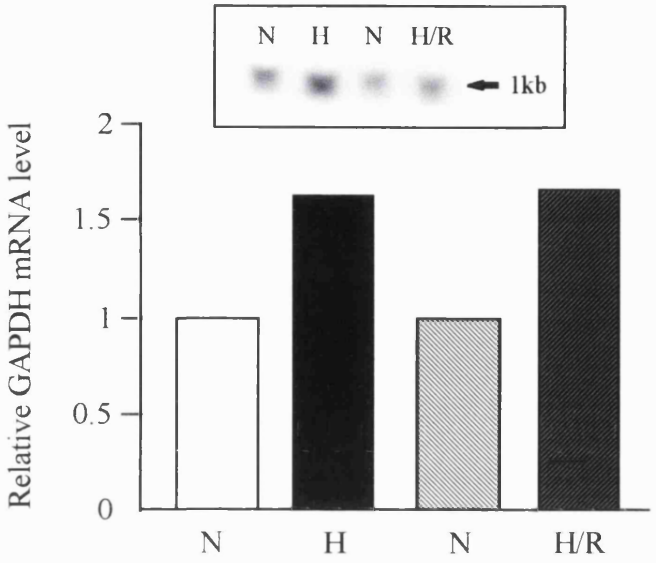


Fig. 2.7 ***Northern Blot analysis of GAPDH mRNA.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21%O_2 (**N**); 24hrs hypoxia 1%O_2 (**H**); exposure to 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxgenation (**H/R**). Scanning densitometry was performed as described in *Chapter 2: 2.3.9.* Relative mRNA levels were calculated as fold over control values (assigned value of 1). The data presented are a representative experiment of 3 repeats. Insert shows representative autoradiogram.

2.5 Discussion

To study the effects of hypoxia on human PTE, a cell culture approach was adopted with cells in monolayer directly exposed to decreased O_2. Although such an *in vitro* system clearly cannot mimic the *in vivo* environment of the kidney, this model permits direct experimental manipulation of human primary PTE without the confounding effects from other cell types. Whilst it has been shown that cells in culture respire anaerobically and may therefore become hypoxia tolerant, previous experiments in our laboratory have demonstrated that PTE remain sensitive to O_2 deprivation for several passages [62,182]. It was therefore considered unnecessary to deplete the

medium of glucose in order to prevent glycolysis, as has been described in other systems [116].

PTE were exposed to a reduced O_2 environment; a method selected in preference to chemical hypoxia which involves the use of metabolic inhibitors such as antimycin A and cyanide to deplete cellular ATP levels [102]. Direct hypoxia avoids unwanted effects of metabolic inhibitors which can have other effects in addition to inhibiting metabolic enzymes; for example cyanide inhibits catalase as well as cytochrome C oxidase, its intended target [102]. Furthermore, the presence of O_2 during chemical anoxia may stimulate the production of ROS, which can interfere with the effects of ATP-depletion thus making data uninterpretable [184].

PTE were subjected to 1%O_2 for 24hrs followed by 24hrs normoxia (21% O_2; referred to as 're-oxygenation'). The degree of hypoxia chosen was based on data showing that *in vitro* exposure of a variety of cell lines to 1% O_2 for a minimum of 16hrs increases EPO mRNA levels and also induces the expression of growth factors such as TGF-β1, PDGF and VEGF [123]. In addition, previous studies in our laboratory have demonstrated that these conditions are sufficient to induce ET-1 mRNA levels in human PTE [62]. A period of re-oxygenation was included as a loose analogy of re-perfusion injury since it is known from *in vivo* models and isolated tubule studies that the damage to cells when oxygenation is restored can be greater than that imposed by the ischaemic injury itself [116-118].

The hypoxia conditions used had no macroscopic effects on cell morphology or viability. Trypan-blue exclusion and lactate dehydrogenase (LDH) release showed no significant difference in cell number or in cell viability suggesting that the level of injury induced does not lead to cell death. Since it is known that ischaemia/reperfusion *in vivo* can alter PTE proliferation [110], it was of interest to examine whether re-oxygenation

post-hypoxia has any effect on PTE *in vitro*. Previous studies in our laboratory reported that 48hrs hypoxia (1% O_2) stimulated proliferation, an observation which may be important in terms of cellular regeneration, post-hypoxia [182]. However, the shorter period of hypoxia (24hrs) used in this study had no effect on PTE proliferation suggesting that the reported proliferative response may be secondary, due, for example, to the autocrine action induction of a hypoxia-induced growth factor.

Whilst the hypoxia conditions used did not alter cell proliferation and viability, these conditions were not without effect and did induce changes in cell metabolism. Protein synthesis was decreased by hypoxia and remained suppressed on re-oxygenation, confirming the findings of other groups that hypoxia impairs PTE protein synthesis [101]. Since protein synthesis is highly dependent on ATP hydrolysis it is likely that decreased synthesis reflects the fall in cellular ATP levels [185]. Although not investigated in this study, it has been reported that total O_2 deprivation (anoxia) can decrease protein phosphorylation in isolated proximal tubules, resulting in altered signal transduction mechanisms [186], demonstrating a further mechanism by which O_2 deprivation can regulate cell behaviour.

As a measure of the effects of hypoxia on the metabolic activity of PTE, GAPDH mRNA expression was examined. This enzyme was chosen as a marker as it is an important component of the glycolytic pathway and can provide some insight into the effects of hypoxia on glycolysis [171]. In addition, it has been reported that the GAPDH gene contains a hypoxia responsive element [127] (discussed in *Chapter 1*). GAPDH gene expression was induced by 24hrs hypoxia and remained high on re-oxygenation, suggesting that O_2 depletion may enhance glycolysis in an attempt to generate sufficient ATP for preservation of cellular integrity. Although not previously reported in PTE, GAPDH upregulation by hypoxia has been described in PAEC subjected to hypoxia

[127]. While PTE have been described as a relatively hypoxia-sensitive cell type [14], the increase in GAPDH mRNA expression would suggest that these cells do possess mechanisms for protection against hypoxic injury.

It is of interest that GAPDH is commonly used as a housekeeping gene for Northern analysis of hypoxia-regulated genes. The data presented here demonstrates that GAPDH is regulated by hypoxia in PTE cells and therefore cannot be used as a 'control' gene in these cells when studying the effects of O_2 deprivation. For this reason, gene expression studies in this project used normalisation to rRNA levels (quantified by densitometry of ethidium-stained gels) to control for variations in RNA loading (*2.3.9*) in Northern blot analysis.

2.6 Summary and conclusions

- Primary cultures of human PTE were established and characterised. An *in vitro* model was developed to study the effects of hypoxia on these cells.

- The period and extent of hypoxia chosen (24hrs, 1% O_2) had no effect morphology, cell proliferation or cell viability of human PTE, suggesting that this degree of O_2 deprivation induces a level of injury which does not cause cell death.

- The degree of hypoxia used was sufficient to decrease protein synthesis and induce GAPDH gene expression, therefore providing some impairment of cellular metabolism. This model was therefore considered appropriate for the examination of ECM regulation in response to hypoxia in PTE.

CHAPTER 3

Hypoxic-regulation of ECM synthesis and degradation in PTE

3.1 Background

As mentioned in *Chapter 1*, TBM thickening in progressive renal disease is attributed to increased synthesis and/or decreased degradation of ECM proteins [66] however the mechanisms underlying altered matrix metabolism in TIF are not well understood. The normal TBM is composed predominantly of collagen IV [22]. This collagen, together with collagens VIII and IX, belong to the non-fibrillar, network-forming sub-class of the collagen superfamily which differ both structurally and functionally from the fibrillar collagens: I, II, III, V and X [187,188]. As with all collagens, collagen IV has a characteristic triple helical structure composed of three polypeptide α chains wound around one another to generate a rope-like collagen molecule of great tensile strength [187]. The collagen IV molecule found in the TBM is composed of heterotrimers containing two α1(IV) and one α2 (IV) chains (ref) although chains α3(IV)-α5(IV) have been described within the glomerular BM and the distal TBM [21,189]. Collagen α chains consist of repeating -Gly-X-Y- sequences, where proline is frequently found in the X position and 4-hydroxyproline in the Y position; the hydroxyproline residues form interchain hydrogen bonds which stabilize the triple helix [189,190]. The individual collagen polypeptide chains are synthesized on ribosomes as pro-α chains with pro-peptides at the amino and carboxyl-terminal domain [187,189]. Pro-α chains undergo hydroxylation of selected prolines and lysines followed by

glycosylation of specific hydroxyl residues before combining to form the triple helix arrangement which is secreted from the cell as pro-collagen [187-189].

Two features of type IV collagen influence the character of the TBM (ref). Firstly, numerous globular regions interrupt the repetitive -Gly-X-Y- sequence of the collagenous domain, enhancing the flexibility of the triple helix [21](*Fig. 3.1*).

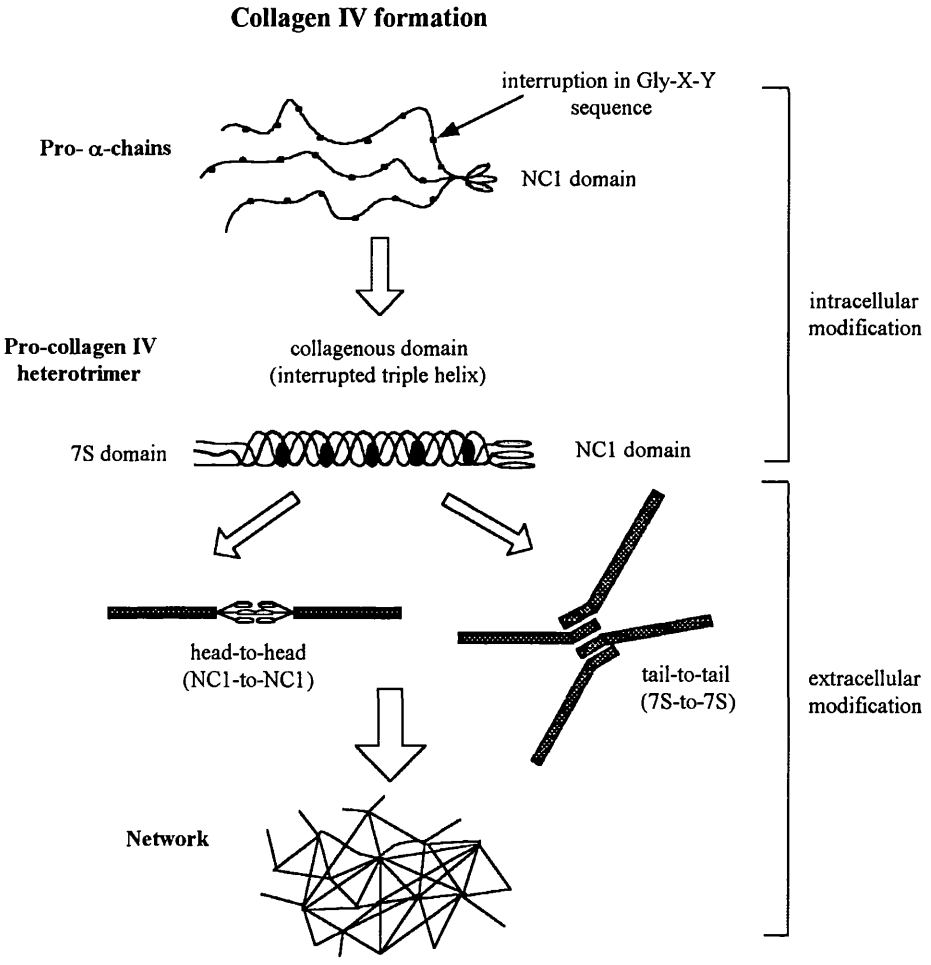


Fig. 3.1 ***Schematic diagram of collagen IV network formation.*** Adapted from [21,187,189,190].

Secondly, unlike fibrillar collagens such as collagen I, type IV pro-collagen molecules retain their pro-peptides on secretion [21]. Procollagen-1 heterotrimers, composed of two α1(I) chains and one α2(I) chain, undergo extracellular proteolytic cleavage and assemble into ordered polymers or collagen fibrils which aggregate into large bundles of collagen fibres [191] (*Fig. 3.2*).

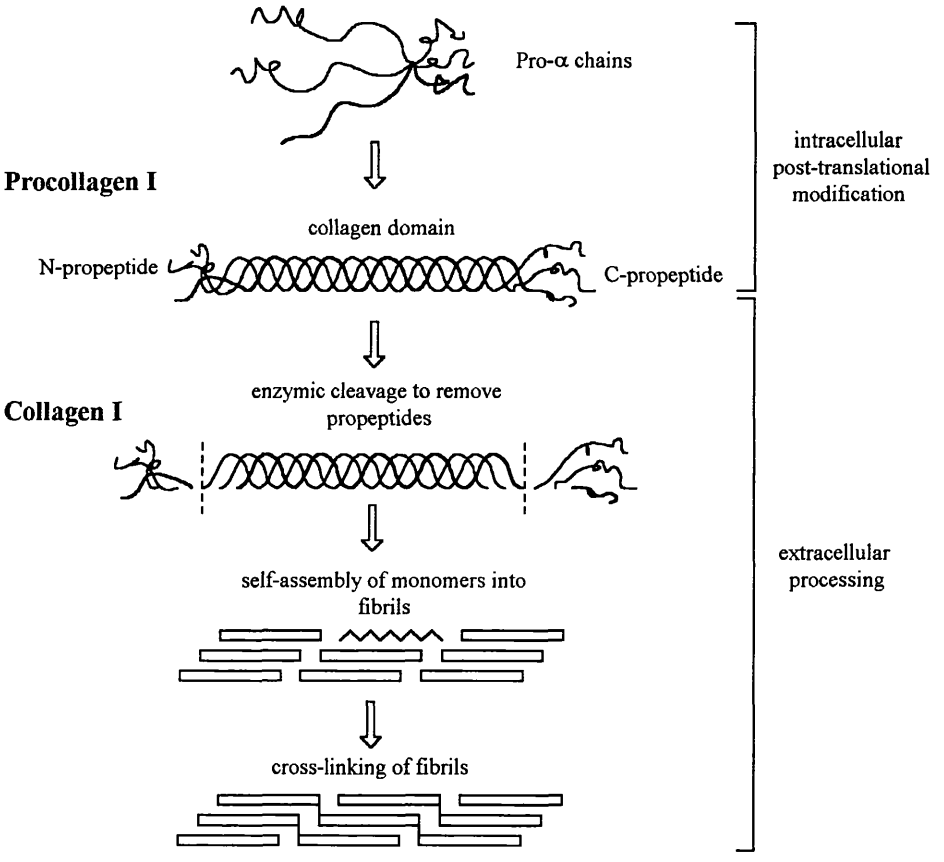


Fig 3.2 ***Schematic diagram of Collagen I bundle formation.*** Adapted from [187,191].

Collagen IV however does not undergo extracellular processing and the pro-peptides are essential for formation of the basement membrane skeleton [21]. Collagen IV molecules form dimers by association of the carboxy-terminal (NC1 domain) and then tetramers by association of their amino-terminal (7S domain) [190]. Stable tetramers

interact to form a sheet-like, multi-layered network which provides the structural backbone of the basement membrane [187,190] (*Fig. 3.1*).

The other major TBM components: laminin, entactin and perlecan, together, with associated proteins such as SPARC (Secreted Protein Acidic and Rich in Cysteine) and osteopontin, form large macromolecular structures within the collagen IV meshwork to create the highly complex, heterogeneous organisation of the basement membrane [22] (*Fig. 3.3*).

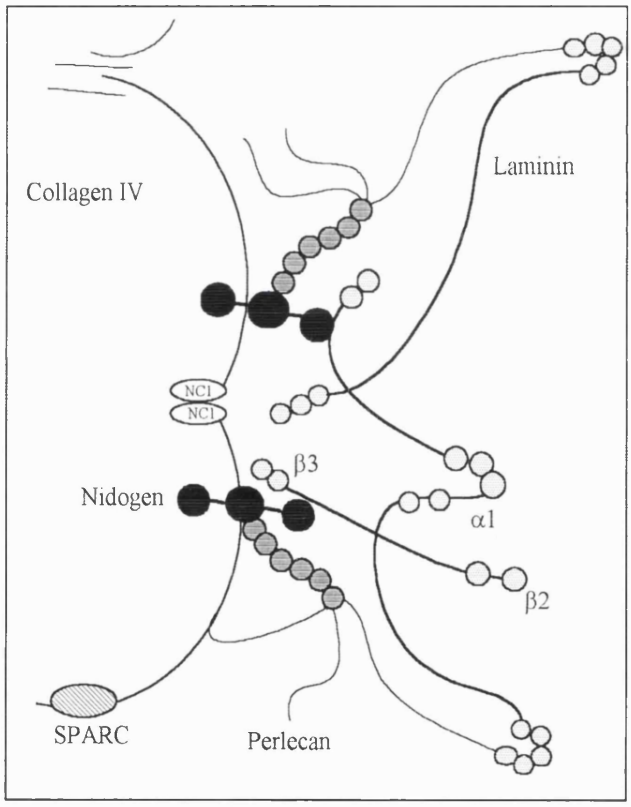


Fig. 3.3 ***Schematic representation of basement membrane assembly model.*** NC1: carboxyterminal domain of collagen IV, α1, β1 and β2 are laminin side chains. Adapted from [22].

The TBM provides structural support for PTE while allowing the passage of ions and macromolecules to pass between the PTE cell and the interstitium [192]. In addition to creating an anatomical barrier, components of the TBM can regulate cell

phenotype, shape, motility and substrate attachment [193,194]. Many of these functions are mediated through interaction of matrix proteins with their associated integrins which not only ensure that cells maintain contact with the underlying BM but provide a means for integrating the ECM with the cytoskeleton [193,194]. Contact of cells with the ECM can profoundly influence the expression of a variety of genes including those encoding transcription factors, growth factors and matrix proteins themselves, providing a mechanism whereby the ECM affects and is in turn affected by the cells that produce it [195,196].

In addition to the direct actions of ECM proteins on cell function, the BM can influence cellular activity by modulating growth factor activity [192,197]. BM proteins can bind and store growth factors such as TGF-β1 and PDGF-BB, which may be released, for example, through the action of matrix-degrading enzymes or activated/inactivated by association with matrix proteins [197]. A number of cytokine/matrix protein interactions have been described; for example TGF-β1 can bind to collagen IV directly [22], SPARC is known to bind PDGF and to block binding of PDGF to its receptor [198] while thrombospondin-1 expression is tightly linked to PDGF-induced cell proliferation in mesangial cells [199]. Thrombospondin-1 has also been described as an endogenous activator of TGF-β in tubulo-interstitial and glomerular disease [200]. In contrast, binding of TGF-β to the proteoglycans decorin or biglycan, directly blocks the activity of this cytokine [201].

In fibrosis, the TBM undergoes both qualitative and quantitative changes [38,49,50]. Increased levels of collagen IV within the TBM have been reported in both *in vitro* and *in vivo* models of fibrosis, however it is unclear whether this collagen is solely responsible for the thickened appearance of the TBM [38,49,84,202]. In addition, it has been reported that collagen IV is often present within the interstitial matrix in TIF,

although the source of this protein in the interstitium has not yet been identified [38,49,202]. Previous studies have demonstrated that normal PTE *in vitro* express low levels of atypical matrix proteins such as collagen I and fibronectin and that production of these may be induced in fibrosis [51,52]. Although collagen I is produced by mesenchymal cells, induction of this protein by PTE has been reported in an *in vitro* model of diabetic nephropathy and in an animal model of experimental autoimmune nephritis [50,51], suggesting that collagen I may contribute to the increased matrix content of thickened TBM in disease. In addition, one can speculate that the appearance of 'interstitial' matrix proteins within the basement membrane raises the intriguing possibility that PTE themselves may be a source of the extracellular proteins which accumulates in the interstitial area in TIF; further studies are required to investigate this possibility.

A number of growth factors that are increased in TIF are known to regulate collagen gene expression and hence may contribute to the accumulation of collagen within the tubulo-interstitium in fibrosis [43,188]. The most potent inducer of collagen gene expression is the premier pro-fibrogenic cytokine TGF-β1 [203,204] (discussed further in *Chapter 4*). TGF-β1 levels are elevated in human progressive renal disease [58,59] and in *in vivo* models of TIF [38,49,202] and it has been demonstrated in animal models that this cytokine mediates the pro-fibrogenic effects of protein-overload and glucose [57,205]. TGF-β can increase steady- state mRNA levels of both collagen I and collagen IV levels in many cell types [203] and in the case of collagen I, this increase may be due to increased gene transcription since TGF-β response elements have been described in the human, mouse and rat collagen I gene promoters [206-208] (discussed in *Chapter 6*). One other possibility is that collagen accumulation may result from an

increase in mRNA stability, as has been demonstrated for collagen I mRNAs some cell types [209].

Hypoxia may be one possible stimulus for ECM accumulation since microvascular damage is a common observation in many fibrotic diseases [10,11]. At present there is no information regarding the effects of reduced O_2 on ECM metabolism in PTE or other tubulo-interstitial cells however one preliminary report (in abstract form) suggests that hypoxia stimulates collagen gene expression in mesangial cells [163]. More data is available from non-renal cell-types, with hypoxia consistently stimulating matrix synthesis; collagen α1(I) mRNA expression and protein levels are elevated in human cardiac fibroblasts after 24hrs hypoxia (2% O_2) [210] while similar experiments with human dermal fibroblasts demonstrated that collagen α1(I) mRNA expression is induced with 12-96hrs of hypoxia (2% O_2); this effect, at least in part, being mediated by a hypoxia-induced increase in TGF-β [164]. In addition, a study of hypoxia-induced hypertension in calves demonstrated matrix re-modelling of the pulmonary arteries of these animals [165] with increased mRNA levels for collagen α1(I), tropoelastin and fibronectin mRNAs within pulmonary artery walls which correlated with increased collagen and elastin fibre volumes within the vessel wall [165]. Whilst these studies all involve non-epithelial cell types in non-renal tissues, they demonstrate that hypoxia can increase the expression of matrix proteins and potentially lead to an accumulation in ECM, providing support for the hypothesis that hypoxia may be a pro-fibrogenic factor for PTE.

In addition to hypoxia, one other regulator of matrix synthesis may be oxidative stress [211]. As mentioned in *Chapter 2*, ARF studies have demonstrated that ROS formation by PTE is increased on re-oxygenation (post-anoxia), causing lipid peroxidation which results in cellular injury [101]. Although it is not yet known whether

similar ROS induction occurs in the re-oxygenation period after hypoxia, it is interesting to note that in human foetal fibroblasts, ROS generation induces collagen I gene expression [212]. The direct effects of ROS on PTE ECM synthesis have not been examined, however an *in vivo* model of ARF demonstrates that re-perfusion induces laminin and fibronectin levels within the TBM, suggesting that ROS may be a stimulus for increased ECM synthesis in PTE [119].

ECM accumulation in TIF is not only the result of increased matrix synthesis but also decreased matrix degradation [5]. The enzymes primarily responsible for degradation of the major basement membrane component, collagen IV are MMP-2 and MMP-9 (gelatinases) [69]. PTE *in vitro* express both active and latent MMP-2 and MMP-9 [74,88]. Although some workers have examined whole tissue mRNA levels of these MMPs in fibrosis, there is little information regarding PTE gelatinase expression in disease [213,214]. Our group has shown that in autosomal dominant polycystic kidney disease (ADPKD), a genetically-determined renal disease with a significant fibrotic component, levels of MMP-2 and MMP-9 in PTE are elevated [74]. In contrast, in rat polycystic kidney disease, PTE mRNA and enzyme activity for MMP-2 is decreased [89]. These results support the general observation that MMP expression in fibrosis appears to be variable and to be disease- and model-dependent, although clearly more studies are required before final conclusions can be drawn.

In normal matrix turnover, a balance exists between matrix degrading enzymes and their inhibitors; while decreased enzyme production or activation will lead to decreased turnover, increased levels of inhibitor will have the same effect [70]. As mentioned in *Chapter 1*, activated MMPs are regulated by the TIMPs [78-80]. Although MMP levels in fibrotic diseases differ according to enzyme and model, many studies have demonstrated that in these diseases, TIMP-1 is upregulated, both at the mRNA and

protein level [5]. The importance of elevated TIMP-1 levels in TIF is currently unclear and is complicated by the fact that TIMPs have many other effects beside inhibition of MMPs, including regulation of cell proliferation, differentiation and apoptosis [215-217]. The recent finding that TIMP-1 knock-out mice develop protein-overload induced TIF in a similar manner to wild-type animals raises further questions regarding the significance of elevated TIMP-1 in TIF [218]. The authors of this study speculate that possible explanations for these results include TIMP-1 genetic redundancy and exacerbation of TIF in the knock-out animals [218]; further studies are required in order to clarify the role of TIMP-1 in TIF.

At present there is no information regarding regulation of MMPs and TIMPs by hypoxia in renal cells and only limited data is available from other cell systems; one report describes an inhibition of MMP-9 in human keratinocytes subjected to hypoxia [166]. Against this background and given that hypoxia may be a common component of renal fibrotic diseases and a stimulus for increased matrix production in some cell types, it was of interest to establish whether hypoxia may be a pro-fibrogenic stimulus in PTE by acting on ECM metabolism. This Chapter investigates whether the hypoxia conditions defined in *Chapter 2* alter matrix metabolism in human PTE.

3.2 Aims

- To investigate the qualitative and quantitative effects of hypoxia on PTE matrix production.
- To determine the effects of hypoxia on ECM turnover in PTE.

3.3 Methods

3.3.1 Collagen Production

Total collagen production was measured by reverse phase HPLC (System Gold, Beckman Instruments Ltd) [219]. Briefly, PTE were grown in 12-well plates (6 replicate wells/treatment) in RPMI 1640 media containing all supplements (see *Chapter 2: 2.3.1*). At confluence cells were transferred to incubation media (RPMI 1640 containing nucleosides, HEPES, transferrin, antibiotic/antimycotic, 23μg/ml proline (BDH-Merck Ltd.) and 50μg/ml ascorbate (Sigma)) for 24hrs. Fresh incubation medium was added to the cells immediately prior to hypoxia (and also to control, normoxic cells). Cells plus media were harvested after 24hrs hypoxia or after a further 24hrs re-oxygenation and the plates stored at -20°C prior to analysis. For HPLC, plates were thawed, the cell layer scraped into the medium and the contents of each well collected. Wells were washed with PBS and the rinse added to the cell suspension. Proteins were precipitated by addition of ethanol (to a final concentration of 67%). The samples were filtered through a 0.4μm filter (type HV; Millipore, Watford, UK) and the filter plus proteins hydrolysed in 6M HCl at 110°C for 16hrs. The hydroxyproline content of the hydrolysates was assessed after separation of the 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) derivative of the imino acid from other amino acid derivatives by HPLC. Quantitation was achieved by measuring the peak area absorbance and comparing it to known standards of hydroxyproline run on the same day where 1 HPLC unit = 1nmol hydroxyproline. Procollagen production is expressed as nmol hydroxyproline/well.

3.3.2 Northern Analysis

RNA extraction and Northern analysis were performed as described in *Chapter 2:2.3.9.* The cDNA for collagen α1(I) (HF677) was obtained from Dr.M.L.Chu (Thomas Jefferson University, Philadelphia, PA, USA) and cDNA for human collagen α1(IV) (HT-21)

from Dr.D.Prockop (Institute of Molecular Medicine, Allegheny Hospital, Philadelphia, PA, USA). cDNA probes for human MMP-2, TIMP-1 and TIMP-2 were a generous gift from Dr.G.Murphy (University of East Anglia, Norwich, UK). Scanning densitometry of autoradiograms was performed as in *Chapter 2: 2.3.9.* Data are expressed as fold change in mRNA level in PTE exposed to hypoxia, calculated from arbitrary densitometry units with respect to the relevant normoxic controls (assigned an arbitrary value of 1).

3.3.3. MMP-2 and MMP-9 protein levels

Gelatinase activity in conditioned medium (CM) was measured by gelatin-substrate gel zymography [24]. Aliquots of CM, containing equal amounts of protein, were concentrated using Microcon-3 (Amicon Ltd, Stone House, Glos, UK) with a molecular weight cut-off of 3kD. Approximately 10-fold concentration was achieved, protein concentration was measured again prior to electrophoresis and adjusted to ensure equal loading. To each sample, an equal volume of 2X sample buffer was added (2X buffer: 125mM Tris pH6.8; 4% SDS; 0.005% bromophenol blue; 20% glycerol) and the samples allowed to stand at room temperature for 15mins. Protein standards (Rainbow markers 14-200kD; Amersham) were diluted in 2X sample buffer containing 1% β-mercaptoethanol (Sigma) boiled for 10mins and cooled on ice for 10mins. Samples were separated by SDS-PAGE under non-denaturing conditions on a 4% polyacrylamide stacking gel and 10% separating gel containing 0.1% gelatin (BDH-Merck Ltd.). The presence of SDS in the agarose gel results in the dissociation of gelatinases from endogenous inhibitors, such as TIMPs, to allow quantitation of both bound and unbound MMP. Gels were electrophoresed for 1-2hrs at 100V and washed twice for 15mins in 2.5% Triton X-100 at room temperature with gentle agitation to remove the SDS and allow re-folding of the enzymes before autolytic cleavage. Gels were rinsed with distilled water, incubated for

30mins in developing buffer (50mM Tris-HCl, pH7.5; 5mM $CaCl_2$; 200mM NaCl, pH7.5) at room temperature and then overnight in fresh developing buffer at 37°C. Incubation in developing buffer activates any latent enzyme which may be present. Gels were stained in 0.5% Coomassie Brilliant Blue G-250 in 30% methanol, 10% acetic acid for 3hrs at room temperature with gentle agitation and then destained in 30% methanol, 10% acetic acid until gelatinase activity was visualised as cleared zones against a blue background. MMP bands generally appeared as a doublet, the upper band indicating the inactive enzyme produced by the cells while the lower band represented the active enzyme produced by proteolytic cleavage of the inactive precursor (*Chapter 1*). Gels were scanned and bands quantified by densitometry as for Northern blots (see *Chapter 2: 2.3.9*). Fold-change in gelatinase activity, with respect to the relevant normoxic controls, was calculated from arbitrary densitometry units, with normoxic controls assigned a value of 1.

3.3.4. ELISA for TIMP-1

TIMP-1 protein in CM was measured using the Biotrak Human TIMP-1 ELISA System (Amersham) according to the manufacturer's instructions. This ELISA system measures total TIMP-1 in the range 3.13-50ng/ml. Briefly, duplicate wells containing 100µl aliquots of standards (0-50ng/ml recombinant, human TIMP-1) or CM samples (diluted 1:5 in assay buffer) were incubated in 96-well plates pre-coated with anti-TIMP-1 antibody for 2hrs at room temperature. Wells were washed 4 times with 'wash buffer' and a peroxidase-labelled anti-TIMP-1 antibody (Amersham) was added to each well for 2hrs at room temperature. Wells were again rinsed 4 times with wash buffer and the substrate 3,3' 5,5'-tetramethylbenzidine (TMB) added to each well for 30mins at room temperature with gentle agitation. Bound TIMP-1 produces a blue colour reaction. The reaction was stopped by the addition of 1M H_2SO_4 which produces a yellow colour change. Absorbance was read at

450nm using a Multiskan MCC/340 plate reader (Titertek, Labsystems, Finland). The concentration of TIMP-1 in CM was determined from the standard curve.

3.4 Results

Extracellular Matrix Production:

3.4.1. Effect of hypoxia on total collagen production

Total collagen production was measured by HPLC of cellular and secreted hydroxyproline, the major amino acid constituent of pro-collagen (ref). Hydroxyproline levels were increased after hypoxia (*Fig. 3.4*) and remained elevated on re-oxygenation, compared to the relevant controls with a significant increase ($P<0.05$) apparent after 24hrs re-oxygenation.

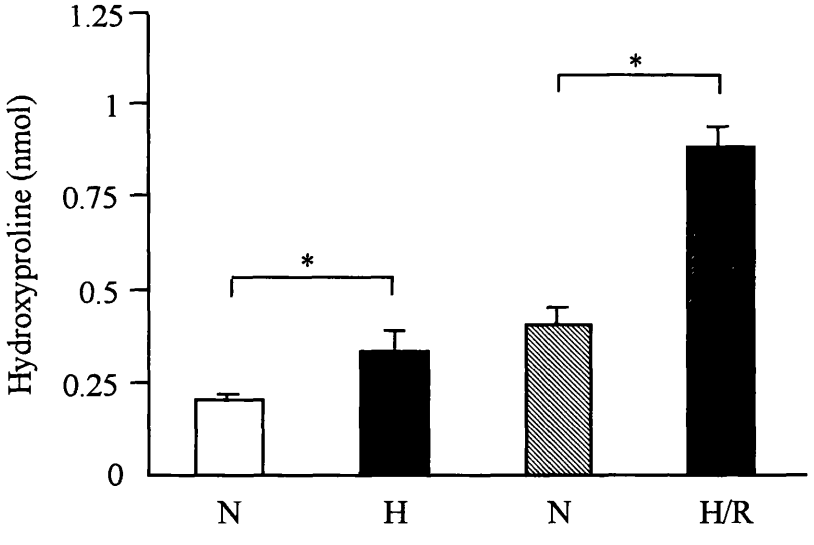


Fig. 3.4 ***Collagen production by PTE exposed to 24hrs hypoxia or 24hrs normoxia.*** Hydroxyproline levels in confluent and quiescent PTE exposed to 24hrs normoxia, 21% O_2 (**N**); 24hrs hypoxia, 1% O_2 (**H**) ; 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). Data are the mean of 2 independent experiments, n=6 wells in each experiment. *$P<0.05$ by unpaired Student's T-Test.

As described in *Chapter 2; Fig. 2.1*, non-collagenous protein synthesis (^{3}H-Phe incorporation) was decreased after 24hrs hypoxia and remained suppressed in the re-oxygenation period, indicating that the stimulatory effect of hypoxia may be selective for specific proteins, including collagen.

3.4.2. Effect of hypoxia on Collagen I and IV mRNA expression

Elevated collagen production in PTE exposed to hypoxia could be due to decreased collagen degradation, increased protein stability or increased gene transcription. Northern blot analysis was performed to establish whether hypoxia has any effect on steady-state mRNA levels of the basement membrane collagen, collagen IV. Under normoxic conditions, PTE expressed collagen α1(IV) mRNA (6.2kb) however levels of gene expression were found to decrease (*Fig. 3.5*), rather than increase, after 24hrs hypoxia (40% of control) and remained low during re-oxygenation (*Fig. 3.5*).

Since collagen IV levels were decreased by hypoxia and therefore could not account for hypoxia-induced collagen production, it was decided to examine expression of the 'interstitial collagen', collagen I, in response to hypoxia. It has been reported that PTE in culture have basal collagen I expression [51] and that collagen I levels in the PTE BM are increased in models of fibrosis [50,51,53]. Under normoxic conditions, PTE expressed low levels of mRNA for collagen α1(I) with transcript sizes of approximately 5.2kb and 4.8kb, although the smaller transcript was not always apparent. Collagen α1(I) expression was stimulated by 24hrs hypoxia and remained elevated during re-oxygenation (*Fig. 3.5*) suggesting that the observed increase in total collagen production is likely to be due to increased collagen I gene expression.

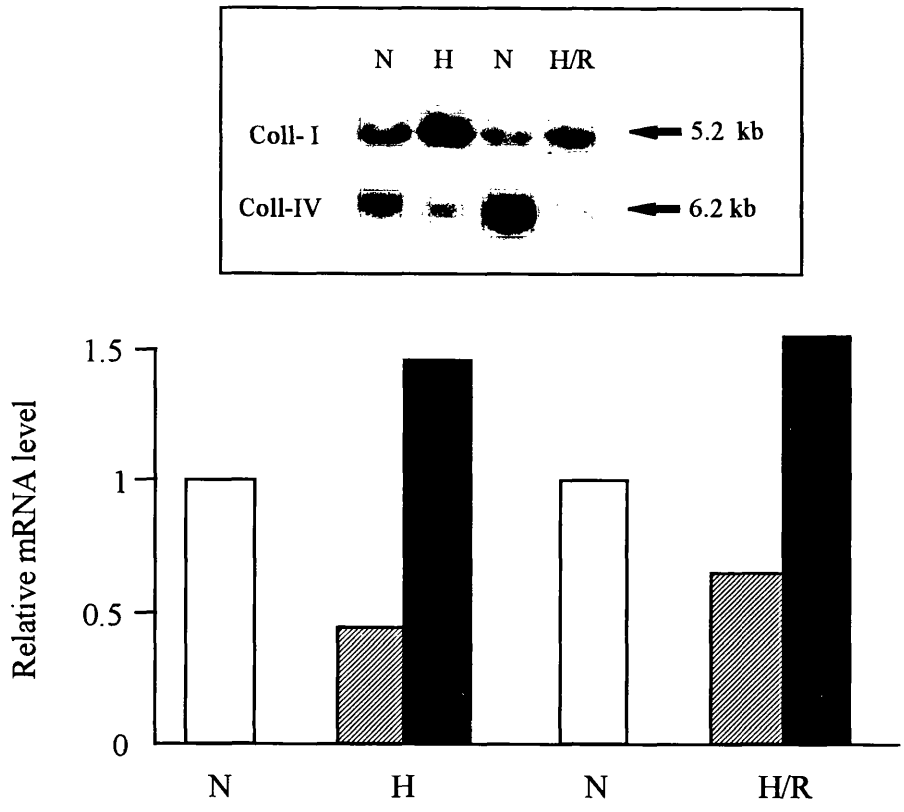


Fig. 3.5 ***Northern Blot analysis of collagen IV (light hatching) and collagen I (dark hatching) mRNA expression of PTE after 24hrs hypoxia and 24hrs re-oxygenation.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21% O_2 (**N**); 24hrs hypoxia 1% O_2 (**H**); 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). Scanning densitometry was performed as described in *Chapter 2: 2.3.9.* Relative mRNA levels were calculated as fold over control values, assigned value of 1 (open bars). The data presented are a representative experiment of 3 repeats. **Insert:** representative autoradiograms.

3.4.3 Time-course of Collagen I mRNA expression in response to hypoxia

In order to establish the period of hypoxia required to increase collagen I mRNA, confluent, quiescent PTE were harvested after exposure to varying periods of hypoxia. Parallel cultures were maintained under normoxic conditions for equivalent time periods. Collagen I mRNA levels appeared to decrease slightly, immediately after the initiation of hypoxia, returning to normoxic levels by 4hrs. Thereafter levels appeared slightly elevated by 8hrs of hypoxia with a marked increase by 24hrs (*Fig. 3.6*).

In an additional experiment, with an intervening time point at 15hrs, levels were greater than at the 8hr time point (data not shown).

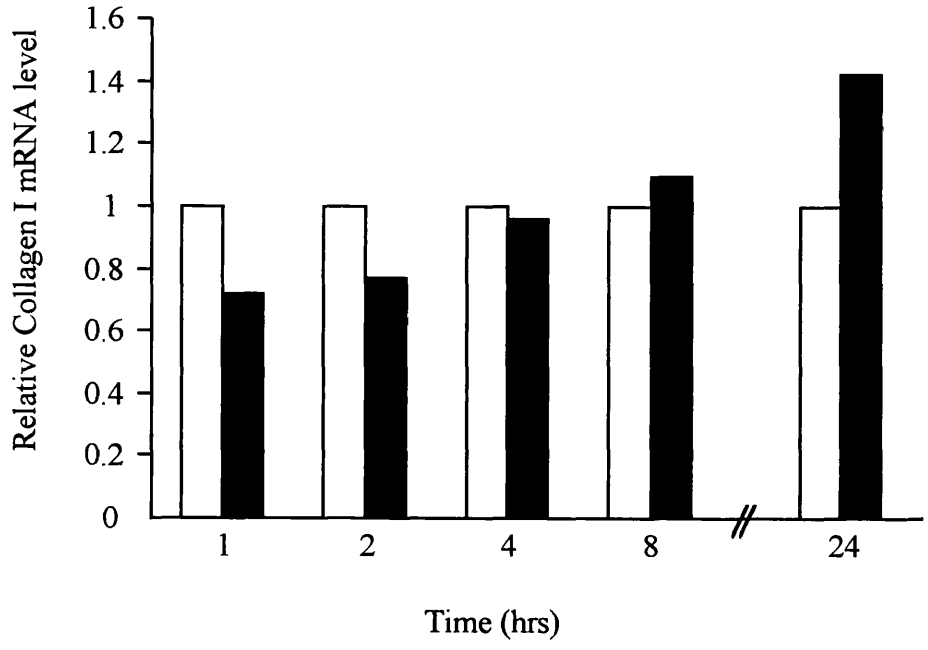


Fig. 3.6 ***Time-course of PTE collagen I mRNA expression in response to hypoxia.*** Total RNA was extracted from PTE exposed to 1,2,4,8 and 24hrs normoxia 21% O_2 (open bars) or hypoxia 1% O_2 (solid bars). Scanning densitometry was performed as described in *Chapter 2:2.3.9.* Relative mRNA levels were calculated as fold vs control values (assigned value of 1). The data presented are a representative experiment.

Extracellular Matrix Degradation:

3.4.4. Effect of hypoxia on gelatinase gene expression and enzyme levels

Since changes in matrix degradation are also thought to contribute to fibrotic matrix accumulation in fibrosis [5], the effect of hypoxia on matrix-degrading enzyme expression was investigated (*Fig. 3.7*). Two major bands of activity were detected in CM from PTE maintained in normoxia for 24hrs; one band at ~72kD representing MMP-2 (gelatinase A) and another at ~92kD representing MMP-9 (gelatinase B). MMP-2 was observed in all PTE-CM; with the active enzyme being the predominant form in CM from normoxic cells.

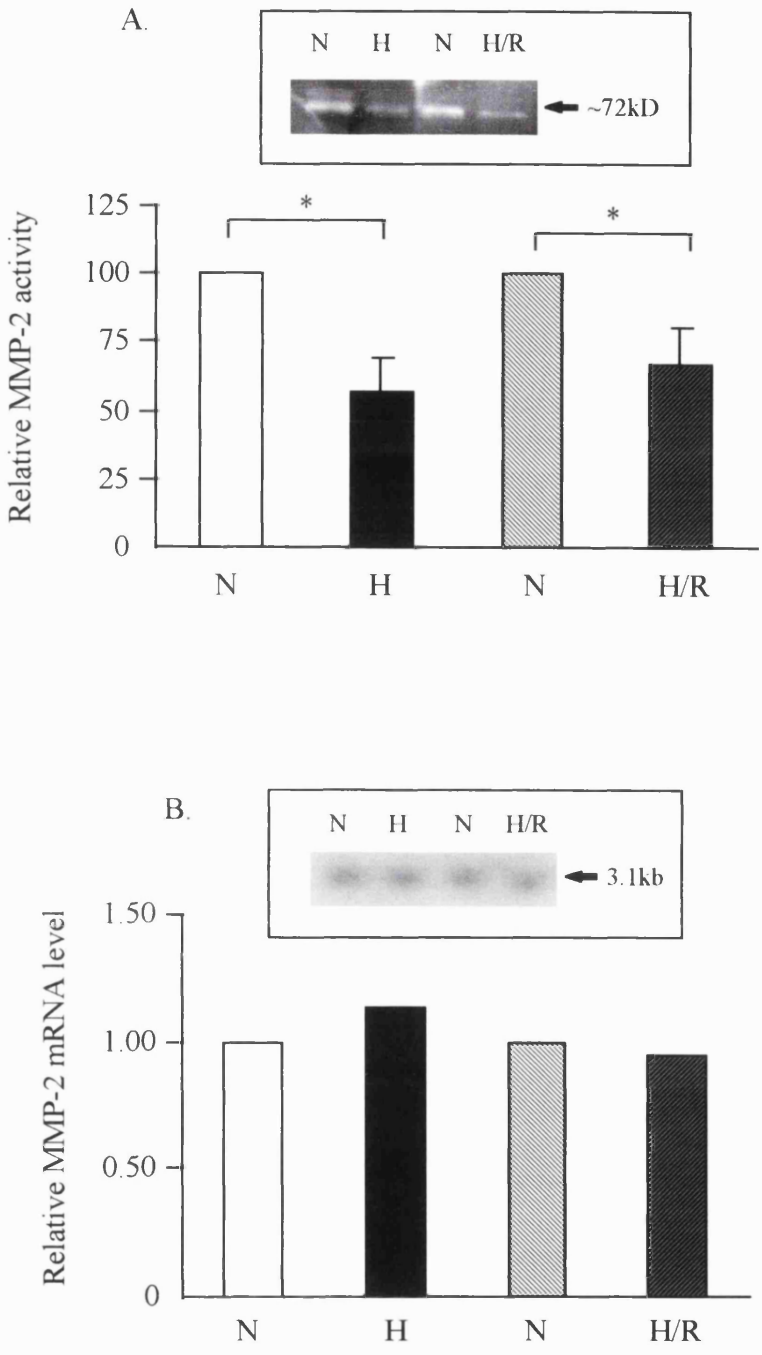


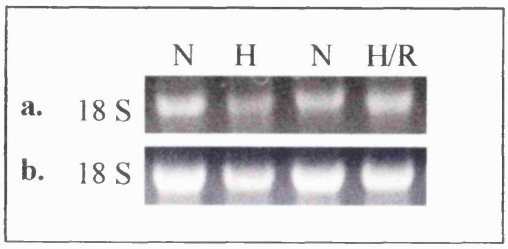
Fig. 3.7. ***A Gelatin-substrate gel zymography of the effect of hypoxia on MMP-2 in PTE.*** CM was collected from confluent, quiescent PTE exposed to 24hrs normoxia, 21% O_2 (**N**); 24hrs hypoxia 1%O_2 (**H**); 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). % change in MMP-2 activity, with respect to relevant controls, was calculated from arbitrary densitometry units with controls assigned a value of 100%. Data are the mean of 3 experiments. **Insert**: Photograph of a representative zymogram. ***B. Northern blot analysis of MMP-2 mRNA expression of PTE after 24hrs hypoxia and 24hrs re-oxygenation.*** Total RNA was extracted from PTE treated as in (***A***). Scanning densitometry was performed as described in *Chapter 2:2.3.9.* Relative mRNA levels were calculated as fold change vs control (assigned value of 1). Data shown are a representative experiment of 3 repeats. **Insert** Photograph of representative autoradiogram.

After 24hrs hypoxia, there was a significant decrease in the active form of MMP-2 (55% ± 13% of control value, assigned 100% (*Fig. 3.7. A*)), which remained suppressed after 24hrs re-oxygenation (65% ± 13.4% of control). Latent enzyme was also suppressed after hypoxia, however this decrease could not be quantified by densitometry as the faint bands obtained were below the sensitivity of the scanner. The decrease in MMP-2 activity after exposure to hypoxia is independent of passage number. The appearance of MMP-9 was more variable but where the enzyme was detected it showed a similar pattern of activity to that described for MMP-2 ie.suppressed by hypoxia (data not shown).

Northern blot analysis demonstrated that PTE expressed a 3.1kb mRNA transcript for MMP-2. MMP-2 mRNA levels were not affected by hypoxia (*Fig. 3.7.B*), suggesting that the decrease in MMP-2 activity is due to post-transcriptional regulation.

3.4.5 Effect of hypoxia on TIMP mRNA and protein

Since hypoxia decreased gelatinase levels, it was of interest to examine whether the MMP inhibitors TIMP-1 and TIMP-2 were also affected. Northern blot analysis of TIMP-1 mRNA levels showed this to be similar in 24hr control and hypoxia-treated cultures, with some increase in the re-oxygenation period (*Fig 3.8*). Secreted TIMP-1 protein levels in PTE-CM (measured by ELISA) increased 20% above control after 24hrs hypoxia (21.5ng/ml vs 25ng/ml). In contrast to TIMP-1, TIMP-2 mRNA levels decreased to approximately 45% of control after 24hrs hypoxia but then rose above the control normoxic value during re-oxygenation (*Fig. 3.8*).



* **Fig. 3.8 (continued).** ***Ethidium bromide-staining of 18S RNA*** . Gels were scanned and intensity of ethidium bromide-staining quantified by densitometry to allow correction for unequal loading. Panels **a.** and **b.** indicate loading of mRNA hybridised with TIMP-1 and TIMP-2 probes respectively (see Fig.3 .8 opposite).

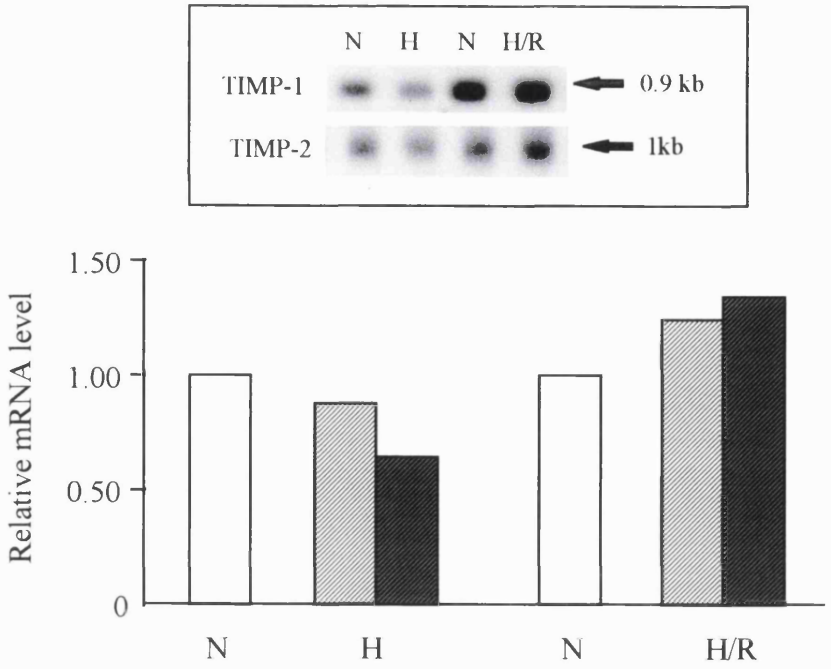


Fig. 3.8 ***Northern Blot analysis of TIMP-1 (light hatching) and TIMP-2 (dark hatching) mRNA.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21% O_2 (**N**); 24hrs hypoxia 1% O_2 (**H**); exposure to 48hrs normoxia (**N**); 24hrs hypoxia followed by 24hrs re-oxygenation (**H/R**). Scanning densitometry was performed as described in *Chapter 2:2.2.9.* Fold over control in TIMP-1 or TIMP-2 mRNA was calculated from densitometry units with controls assigned the value 1 (open bars). Data are representative experiments of 2 repeats. **Insert:** a representative autoradiogram.

* Fig. 3.8 continued on opposite page.

3.5 Discussion

In the normal kidney, the TBM separates epithelial cells from neighbouring cell types and is essential for cell function and interaction [21,23]. The normal BM is maintained by a balance between synthesis and turnover [5,70] which is disrupted in fibrosis, leading to thickening of the TBM with altered composition [50,51]. The mediators of changes in ECM metabolism in fibrosis have not been identified, but it is hypothesised that hypoxia has a role in the pathogenesis of progressive renal disease [10,90]. The studies described in this chapter investigate the effects of hypoxia on ECM metabolism in PTE.

Initial investigations examined the effect of hypoxia on collagen production. Total cellular and secreted collagen were measured by hydroxyproline content, the major amino acid constituent of collagen [220]. HPLC analysis of hydroxyproline content was chosen as the most suitable technique since it has been shown to have greater sensitivity and accuracy than incorporation of radiolabelled proline or ELISA methods [219]. In addition, analysis of hydroxyproline content by HPLC allows simultaneous measurement of both collagen synthesis and degradation (although in these studies, high hydroxyproline levels in RPMI-1640 growth medium precluded the measurement of degradation). The HPLC technique is based on the assumption that collagen is the only hydroxyproline-containing protein synthesised by cells [219]. Other hydroxyproline-containing proteins which may be present are the complement protein Clq and elastin but these are unlikely to interfere with the assay since they contain less hydroxyproline and the rates of production of these proteins are much lower than those of collagen [219].

Whilst measurement of collagen production by incorporation of ^{3}H-proline is commonly used, there is often an initial lag period in incorporation which can lead to an underestimate in collagen levels [219]. The ELISA method, although rapid to perform, can also be inaccurate since collagens are not highly antigenic. At the time these studies were performed, suitable collagen I antibodies were not widely available and there were concerns regarding cross-reactivity between antibodies of different collagen types.

Hypoxia markedly increased collagen production by PTE which increased further on re-oxygenation. This increase in total collagen levels occured despite an overall decrease in non-collagen protein synthesis (*Chapter 2:2.4.3*), suggesting differential effects of hypoxia on collagen and non-collagenous proteins. Although not investigated in this study, it may also be of interest to examine whether hypoxia has any effect on the structure of collagen protein. During pro-collagen processing, the

hydroxylation of proline and lysine requires the presence of molecular O_2 and it has been reported that O_2-depletion results in under-hydroxylated collagen [221]. Failure of collagen hydroxylation, as a result of hypoxia, produces a weakened collagen structure in wounds. Although the degree of hypoxia imposed in this study (1% O_2) is not low enough to inhibit the action of enzymes responsible for collagen hydroxylation, under-hydroxylation may have some relevance to PTE *in vivo*, since collapsed proximal tubules are often observed in advanced TIF [222].

HPLC analysis of hydroxyproline measures total collagen levels but does not distinguish between the different collagen classes, therefore mRNA levels of the major basement membrane collagen, collagen IV were measured to assess whether increased collagen production was due to an increase in collagen IV. Surprisingly, collagen IV mRNA levels were found to decrease after hypoxia and remained suppressed even after restoration of normal O_2 concentrations. Since it has been reported that PTE in TIF may express the 'interstitial' collagen, collagen I [26], levels of this collagen were examined. In contrast to collagen IV, collagen I mRNA levels were markedly increased by hypoxia; the time-course experiment demonstrating that this response occurs early after the induction of hypoxia, increasing after only 8hrs of decreased O_2. Collagen I mRNA levels on re-oxygenation were slightly higher than those during hypoxia and continued to increase for up to 72hrs of re-oxygenation (data not shown). Further experiments are required to examine whether the sustained increase in collagen I, with re-oxygenation, is due to the persisting effect of hypoxia, or whether re-oxygenation *per se* induces collagen I mRNA levels, perhaps via ROS generation.

The induction of collagen I by hypoxia suggests that increased collagen production is due to the stimulation of 'interstitial' rather than BM collagen. Since matrix proteins themselves act as regulators of cell growth, differentiation and function,

the presence of atypical collagen in the TBM will likely have profound effects on tubular cell behaviour [193,194]. In addition, the increase in collagens type I and III in the fibrotic interstitium has been largely attributed to interstitial fibroblasts [5]; however the increased expression of interstitial collagens by PTE raises the possibility that in addition to causing thickening of the TBM, PTE may also contribute to interstitial matrix accumulation [26].

The expression of 'interstitial' proteins in the basement membrane lends support to the theory that in fibrosis, PTE may undergo trans-differentiation to a more mesenchymal phenotype [223]. In murine models of anti-basement membrane disease, PTE express the fibroblast specific protein (FSP-1) and the mesenchymal cytoskeletal marker, vimentin [224]. Also, in isolated tubule models of ischaemia/reperfusion, PTE induced to proliferate in order to re-populate the damaged tubule, show strong immunostaining for vimentin [225]. Further support for epithelial transdifferentiation is provided by a report that in the 5/6 nephrectomy model of TIF, PTE express the myofibroblast marker, α-smooth muscle actin [226]. PTE cells are unique in that they are the only tubular epithelia developmentally derived from the mesenchyme; the above studies suggest that in response to injury, these cells may revert to an embryonic phenotype [227].

Since there is accumulating evidence that PTE in TIF undergo transdifferentiation, preliminary experiments were undertaken to investigate whether hypoxia may be a stimulus for this switch in phenotype. However, mouse PTE exposed to hypoxia (1% O_2 for 24hrs) did not express vimentin or α-SMA protein levels and by Western blot analysis showed no increase in FSP-1 mRNA expression (data not shown). One possibility is that collagen I may be an initiating trigger for transdifferentiation, setting in train a cascade of events which only manifest with more prolonged exposure to

hypoxia. Alternatively, it may be that the effect of hypoxia on collagen I is specific to this protein and not involved in the transdifferentiation process.

The stimulation of collagen I mRNA levels with hypoxia has been described in mesangial cells (in abstract form) [163] and in some non-renal cells however [164-166], the finding in this study that collagen IV is also regulated by hypoxia, has not been described previously. Although outside the scope of this study, the suppression of collagen IV is potentially of interest since to date comparatively few genes that are negatively regulated by hypoxia have been reported; genes that are suppressed by hypoxia include the enzymes phosphoenolpyruvate carboxykinase [141], glucose transporter-2 [131], endothelial nitric-oxide synthase [142] and angiopoietin [143] (*see Table 1.3*). In these examples, as with collagen IV expression in the present study, mRNA levels were measured by Northern analysis. It is important to bear in mind that Northern analysis measures steady-state levels of mRNA and therefore does not distinguish between altered gene transcription and changes in mRNA stability. Hypoxia is known to act on both gene transcription and mRNA stability in some positively regulated genes (such as VEGF), however the mechanisms by which hypoxia decreases steady-state collagen IV mRNA levels remain to be elucidated [161].

The observation that hypoxia increased ECM synthesis in PTE together with the finding in *Chapter 2* that PTE exposed to hypoxia have increased attachment to the substrate, raised the possibility that hypoxia may also have effects on PTE integrin expression. Although outside the scope of the current study, it is worth noting that in preliminary experiments PTE subjected to 1% O_2 for 24hrs showed increased immunostaining for the $\alpha v\beta 3$ integrin (vitronectin/fibronectin receptor) which returned to control levels on re-oxygenation. Examination of the αv and $\beta 3$ sub-units at the mRNA level demonstrated that expression of these sub-units is also induced by hypoxia

(3-fold and 1.3-fold respectively) [228]. This demonstrates that in addition to increasing PTE ECM, hypoxia concomitantly stimulates integrin expression. Since integrins not only mediate adhesion to matrix components, but also function in signal transduction, cell growth, differentiation and survival, increased $\alpha v\beta 3$ expression in hypoxia may be of importance; the significance of altered PTE integrin expression in hypoxia awaits further investigation.

Matrix accumulation in TIF occurs not only through increased matrix synthesis but also as a result of decreased ECM degradation [55]. The major physiological regulators of matrix turnover are the MMPs, with enzyme activity regulated by the TIMPs [83]. Gelatinases are likely to be involved in PTE BM turnover as their primary substrate is collagen IV [70]. Gelatinase levels in PTE CM were measured by gelatin-gel zymography, a technique which allows visualisation of both active and latent enzyme forms and separates MMPs from any bound inhibitors. It has been suggested that MMP-2 is constitutively expressed while MMP-9 is the inducible gelatinase, however the data presented here demonstrate that, *in vitro*, hypoxia suppresses activity of both MMP-2 and MMP-9 in PTE cells and that this decrease is sustained even when cells are returned to normoxia. The effect of hypoxia on MMP-9 is in accordance with decreased enzyme levels reported in human keratinocytes [166], however hypoxia-mediated changes in MMP-2 have not been previously described in any cell type. Regulation of MMP-2 activity in PTE appears to be post-transcriptional since mRNA levels were not affected by hypoxia. Thus the data point to translational or post-translational regulation of enzyme levels, as a result of decreased protein production, decreased protein stability or as the result of altered enzyme secretion as a consequence of low energy availability in hypoxia.

PTE secreted TIMP-1 under normoxic conditions and levels were increased after 24hrs hypoxia. However, there was no effect on mRNA expression after 24hrs hypoxia suggesting post-transcriptional regulation of the inhibitor in this period. Studies from our group have demonstrated that in contrast to the effect of hypoxia on TIMP-1 expression in PTE, TIMP-1 mRNA levels are increased in renal interstitial fibroblasts subjected to the same degree of hypoxia ($1\%O_2$ for 24hrs) [229]. This suggests that the effect of hypoxia on the TIMP-1 gene may be cell-type dependent, as has been demonstrated for other O_2-sensitive genes such as EPO and TGF-β1 [123].

TIMP-2 mRNA was also expressed by normoxic PTE. In contrast to TIMP-1, levels were transiently suppressed by hypoxia, indicating a differential effect of hypoxia on the two inhibitors. Both TIMP-1 and -2 mRNA levels were increased on re-oxygenation, implying either a delayed increase in these inhibitors with hypoxia or a specific effect of re-oxygenation [211]. MMP-2 is known to complex with TIMP-2 [69], and during hypoxia, MMP-2 levels and TIMP-2 were co-ordinately decreased. However in the re-oxygenation phase, TIMP-2 levels rose, suggesting that the balance between enzyme and inhibitor was lost in this period.

The data in this chapter demonstrate that 1% O_2 for 24hrs alters ECM synthesis and turnover in PTE cells. It is not clear at present whether the changes occur in parallel or sequentially whereby the effect of hypoxia on one parameter triggers a change in another aspect of the response. For example, the collagen I time-course demonstrates that hypoxia-induced collagen I expression is a relatively early event and could potentially lead to a rapid change in the composition of the BM, perhaps influencing the regulation of other parameters; further experiments are required to clarify this point.

3.5 Summary and conclusions

- Hypoxia stimulates PTE collagen production with increased mRNA expression of collagen I, an isoform more commonly associated with interstitial matrix rather than the TBM.
- Hypoxia decreases matrix turnover via decreased gelatinase activity and increased expression of TIMPs.
- Similar qualitative and quantitative changes in the TBM in response to hypoxia *in vivo*, would alter both tubular cell function and paracrine interactions with other cell types.

CHAPTER 4

The Effects of Hypoxia on PTE Matrix Metabolism are not Mediated by a Stable, Secreted Factor

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4.1 Background

The studies described in *Chapter 3* demonstrate that hypoxia alters PTE matrix metabolism towards a fibrogenic phenotype, increasing matrix production and decreasing activity of enzymes which regulate turnover. The next stage in the project was to determine how these effects are mediated. One mechanism by which hypoxia can alter gene expression is by induction of secreted factors such as growth factors which then act via autocrine pathways to induce or suppress the relevant genes [62,123,124]. A large number of growth factors are associated with fibrosis and many of these are known to be induced by hypoxia [230-232] (*Table 4.1*). TGF-β1 is the most potent fibrogenic cytokine in the kidney [201], is known to be hypoxia-inducible in several cell types [123] and is produced by PTE [61,233] thus it was considered the most likely mediator of hypoxia-induced changes in ECM metabolism of PTE. Other potential candidates which are produced by PTE include PDGF, ET-1 and VEGF, all of which can be induced by hypoxia and are upregulated in TIF [37,62,230] (*Table. 4.1*).

Growth factors induced in TIF	Produced by PTE	Inducible by hypoxia	Refs
TGF-β1	+	+	[61,123]
PDGF-BB	+	+	[37,123]
VEGF	+	+	[230,124]
ET-1	+	+	[62]
TGF-α	+	ND	[36]
IGF-1	_	ND	[231]
TNFα	+	+	[42,137]
IL-6	+	+	[37,139]
IL-1	-	+	[36,140]
aFGF	-	ND	[232]

Table 4.1 ***Potential mediators of the hypoxia-induced effects on PTE matrix metabolism..*** Listed in the table are growth factors associated with TIF; + denotes those factors which are produced by PTE and are known to be induced by hypoxia in other cell types. ND=no data.

TGF-β1 is one of a family of 3 TGF-β isoforms which are present in mammals [54]. TGF-β1, -β2 and -β3 are all found in the kidney but distribution and level of expression are species- and cell type-dependent; human PTE mainly express TGF-β1 and -β2 whilst murine PTE only express TGF-β1 and -β3 [62]. Although the biological properties of these three isoforms are interchangeable *in vitro*, TGF-β1 has been strongly implicated in fibroses of many different organs, including the lung, liver and kidney [56]. TGF-β1 is a ubiquitous growth factor which has pleiotropic actions on many cellular processes, including differentiation, proliferation and ECM metabolism [204].

Unlike other cytokines that are fully active when secreted from cells, TGF-β1 (and other TGF-β isoforms) is released as part of a latent complex which requires proteolytic cleavage to release the active cytokine [55]. The latent complex is composed of the mature TGF-β homodimer and the latency-associated peptide (LAP) which is

comprised of the NH_2-terminal region of the precursor peptide [55] (*Fig.4.1*). In some tissues, this complex exists in association with a third gene product, the latent TGF-β-binding protein (LTBP) (*Fig.4.1*).

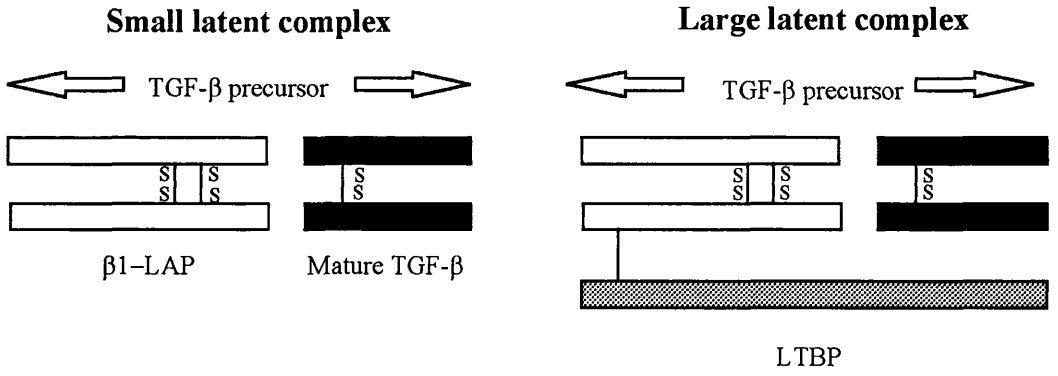

Fig.4.1 ***Schematic drawing of TGF-β complexes.*** Release of active cytokine requires cleavage of the TGF-β precursor molecule from associated binding proteins. Adapted from [55].

Latent forms are differentially secreted in the normal human kidney; tubular epithelial cells secrete the small latent complex (mature TGF-β + LAP) while parenchymal and arteriolar cells of the glomerulus secrete the large latent complex (mature TGF-β +LAP+LTBP) [205]. Biological activation of TGF-β occurs by cleavage of the binding proteins from the mature TGF-β dimer, this can be achieved *in vitro* by acidification, alkalinization, heating or by treatment with urea [56]. The physiological activators have not been identified but are thought to include plasmin, cathepsin D, furin, thrombospondin-1 and sialidase [55,56,234].

Active TGF-β binds to at least four membrane receptors: types I, II and III that exist on virtually all cells studied [204]. The type I and type II receptors are transmembrane serine-threonine kinases that interact with one another to facilitate

receptor signalling [204]. The type III receptor, also called betaglycan, is a proteoglycan which has no signalling structure. Betaglycan is found in both soluble and membrane-anchored forms; the soluble form is present in both serum and ECM while the membrane-bound form acts to present TGF-β to the other receptors [204]. The effects of TGF-β on the synthesis and deposition of ECM are thought to be mediated by the type I receptor whilst the effects on cell growth occur via the type II receptor [204]. A number of accessory proteins such as endoglin [235] bind to these receptors and with specific signalling molecules to form signalling receptor complexes. Recent data has shown that the actions of TGF-β are mediated through members of a family of signalling molecules termed Smads which can positively and negatively mediate TGF-β actions [236].

Secreted, latent TGF-β1 may exist in soluble form or bound to the ECM [205] via small proteoglycans such as decorin, biglycan and fibromodulin [55] . The matrix acts as a storage site for the inactive complex and the proteoglycans are considered natural inhibitors since their association with TGF-β reduces bioavailability [56]. Regulation of bioactive TGF-β1 is therefore complex and occurs at multiple levels: assembly and activation of the latent complex, modulation of receptor expression and association of TGF-β1 with inhibitors [54-56]. At the level of the gene, transcription is regulated by growth factors and other stimuli and the ability of TGF-β1 to auto-induce production greatly amplifies its biological actions [237]. Moreover, active TGF-β1 promotes not only its own gene transcription but also that of the activating cleavage enzyme, resulting in increased secretion of both the active and latent forms [234].

Elevated TGF-β1 levels have been reported in a variety of human renal diseases and in experimental models of TIF [5](*Table 4.2*) and one study reports of a progressive

increase in mRNA levels for receptors I and II during the course of adriamycin nephropathy [238].

Human TIF Diseases	Animal Models
IgA nephropathy	Protein-overload proteinuria
Diabetic nephropathy	Aminonucleoside nephrosis
Diffuse proliferative lupus nephritis	Adriamycin-induced nephrosis
Chronic renal allograft rejection	Chronic anti-Thy-1 glomerulonephritis
HIV nephropathy	Obstructive uropathy
Polycystic kidney diease	Hypercholesterolemia
Chronic glomerulonephritis	Biobreeding rat
Obstructive nephropathy	Non-obese diabetic mouse
	Streptozotocin-induced diabetes
	Anti-GBM nephritis
	Murine Lupus nephritis

Table 4.2 ***Human TIF diseases and animal models associated with elevated TGF-β levels.*** This list includes diseases and animal models where the increase in TGF-β is at either the protein and/or mRNA level. (adapted from [5]).

Although the source of increased TGF-β1 in TIF may be disease- and model-dependent, elevated TGF-β1 has been described in interstitial fibroblasts and inflammatory macrophages [5] and many studies report an increase in TGF-β1 mRNA expression and/or protein in PTE [38,61,233]. In three models of DN: streptozotocin-induced diabetes in rat, the Biobreeding rat and the non-obese diabetic mouse, up-

regulation of TGF-β1 protein is observed in the tubular epithelium very early in disease [205]. The induction of TGF-β1 in *in vivo* models of DN is reproducible in tissue culture; in one study, PTE expression of TGF-β1 mRNA and bioactivity are significantly increased within 48hrs of exposure to high glucose [61]. The increase in TGF-β1 expression in PTE is not unique to DN since upregulation is also described in other models of TIF including protein-overload proteinuria [38], an *in vivo* model of hypercholesterolemia [239] and in proximal tubules isolated from rats with acute PAN nephrosis which demonstrate a 2-fold increase in TGF-β1 mRNA [57].

Increased TGF-β1 in TIF is associated with many aspects of disease including inflammation, cell proliferation, cellular hypertrophy and increased ECM accumulation [54-56]. TGF-β1 modulates proliferation in virtually every cell type [60], generally inhibiting the growth of epithelial cells such as PTE [44]. The effect of TGF-β1 on proliferation is not only cell-type dependent but can also be concentration-dependent within the same cell [240]. This bimodal effect is demonstrated in human foreskin fibroblasts, aortic smooth muscle cells and chondrocytes where maximal cell proliferation is observed when cells are treated with 0.1ng/ml TGF-β1 but at both lower and higher concentrations (0.02, 0.05, 1.0 and 5.0ng/ml), TGF-β1 is less mitogenic [240]. In these cell types, the stimulatory effect of TGF-β1 on proliferation is attributed to TGF-β1-induced PDGF-AA, demonstrating that although TGF-β1 can have direct effects on cellular function, in some cell types, its actions involve the intermediary action of other potent growth factors [240]. Since TGF-β1 itself is induced by cytokines such as PDGF and EGF [237], it is likely that many complex autocrine and paracrine interactions exist among growth factors to tightly control their expression.

Although TGF-β1 inhibits proliferation of many cell types, it enhances mRNA and protein synthesis and thus can promote cellular hypertrophy [241]. TGF-β1 has a

potent hypertrophic effect on tubular cells in culture; NRK-52E (a rat kidney epithelial cell line) grown in the presence of TGF-β1 (10^{-10}M) have enhanced protein synthesis while cellular proliferation is unchanged [242]. In this study, the hypertrophic response was even greater when cells were simultaneously treated with TGF-β1 and EGF; illustrating the synergistic action of TGF-β1 in combination with another growth factor [242]. Effects of TGF-β1 on cell growth have been extensively studied in DN where there is increase in kidney size with marked hypertrophy of both tubular and glomerular elements [56]. In an animal model of diabetes, treatment with anti-TGF-β1 antibody reduced kidney weight by 50% suggesting a cause-and-effect relationship between TGF-β1 and hypertrophy [205].

In fibrosis of diverse organs, increased TGF-β1 is associated with ECM accumulation [54, 204]. TGF-β1 is unique in its ability to increase the deposition of ECM by three simultaneous mechanisms: 1) TGF-β1 acts directly to stimulate the synthesis of many matrix molecules including fibronectin, collagens and proteoglycans; 2) TGF-β1 blocks the degradation of matrix by inhibiting the secretion of matrix-degrading proteases and inducing the production of protease inhibitors; 3) TGF-β1 modulates the expression of integrin matrix receptors on cells in a manner that facilitates cell-matrix adhesion and matrix deposition [201]. These effects have been demonstrated in both renal cells and cells derived from other organs [54,204]. TGF-β1 action appears to differ according to cell type; for example collagen induction by TGF-β1 in dermal and lung fibroblasts is less than that observed in 3T3 fibroblasts [209].

In response to TGF-β1, human PTE not only increase production of the BM protein collagen IV [243] but also upregulate fibronectin synthesis [45]. Although PTE are known to produce basal levels of fibronectin, this protein is more commonly produced by fibroblasts in the tubulo-interstitium [5], suggesting that treatment with

TGF-β1 may affect cell phenotype. Furthermore, human PTE treated with TGF-β1 preferentially up-regulate synthesis of the extra domain A (EDA) splice variant of fibronectin, an isoform which appears in the tubulointerstitial compartment early in TIF [244]. Another 'abnormal' matrix protein induced by TGF-β1 in PTE is collagen I; NRK-52E cells demonstrated an 8-fold increase in collagen α1(I) mRNA levels after exposure to TGF-β1 [44]. The cellular mediators involved in the enhanced matrix production by TGF-β1 have not been defined although transcriptional regulation is a key feature of the effect of this growth factor on collagen I and collagen IV [56]. Induction of the rat collagen α1(I) gene is mediated via the interaction of a TGF-β1-induced nuclear-factor-1 (NF-1) to a sequence-specific DNA-binding site in the collagen I promoter [208]. In addition to transcriptional control, TGF-β1 can also stabilise collagen α1(I) message, as has been described in confluent 3T3 cells and human dermal fibroblasts [209].

The effects of TGF-β1 on matrix degradation in PTE have not been investigated; in proteinuric rats, increased TGF-β1 within the tubulo-interstitium correlates with elevated levels of PAI-1 and TIMP-1, but blocking studies have not yet been performed to prove an association [38]. Similarly, in the rat PAN nephrosis model of TIF, induction of TGF-β1 occurs simultaneous with increased TIMP-1 mRNA, but again no causation has been demonstrated in this model [49], although TGF-β1 is known to increase TIMP-1 expression [67]. The effects of TGF-β1 on ECM degradation have been studied in more detail in other cell types and show that TGF-β1 is a potent regulator of components of both the MMP and plasminogen/plasmin pathways [245,246]. Within the glomerulus, the plasminogen/plasmin cascade is central to ECM degradation and *in vitro* experiments have shown that mesangial cells exposed to TGF-β1 in culture induce PAI-1 and decrease secretion of plasminogen activators [247].

While protease inhibitors are often induced by TGF-β1, proteases themselves may be induced or repressed; treatment of human cervical epithelial cells with TGF-β1 induced both MMP-2 and MMP-9 [67] while corneal stromal cells had decreased levels of MMP-1 and MMP-3 on exposure to TGF-β1 [248]. Studies investigating the effects of TGF-β1 on human fibroblasts have shown that in addition to transcriptional changes, TGF-β1 can also alter post-transcriptional events; in this cell type, increased TIMP-1 mRNA was due to increased gene transcription whereas elevated MMP-2 mRNA was a result of both transcriptional changes and increased message stability [249].

One other fibrogenic effect of TGF-β1 is the induction of integrin expression [203]. It has been demonstrated that exogenous TGF-β1 can up-regulate expression of the αvβ3 integrin in a human lung fibroblast cell line and αLβ2 in a human monocyte cell line [250]. Although the effects of TGF-β1 on PTE integrin expression have not been investigated, *in vitro* experiments with glomerular cells have shown that TGF-β1 can induce expression of integrins that bind fibronectin and type I collagen [251]. In glomerular cells, this effect of TGF-β1 on cell-matrix interactions is an important modulator of both the quantity and composition of the ECM; although not yet demonstrated, similar effects on PTE cells may contribute to the thickening and altered composition of the basement membrane.

In vivo studies by Border et al have demonstrated that in a rat model of glomerulosclerosis, the administration of TGF-β1 neutralising antiserum suppresses ECM deposition, hence demonstrating a link between TGF-β1 and glomerulosclerosis [252]. Until recently, no such causal relationship had been demonstrated for TIF, however one preliminary study (published in abstract form) suggests a crucial role for TGF-β1 in TIF [253]. In this model, TIF was induced by ureteral occlusion. The ureters of animals were either injected with HVJ control liposomes or HVJ -liposomes

containing TGF-β1 antisense oligo-deoxynucleotides (ODN) which target interstitial fibroblasts. The control obstructed animals had elevated TGF-β1 levels and developed TIF, while TGF-β1 antisense ODN treatment reduced TGF-β1 expression and suppressed TIF [253]. These results implicate TGF-β1 in the development of TIF, however it is unlikely that TGF-β1 alone is responsible for fibrosis since it has been demonstrated that the chronic administration of human recombinant TGF-β1 to rats and rabbits results in glomerulosclerosis but has no effect on the tubulo-interstitium [5]. Clearly further studies are required to clarify the role of TGF-β1 in TIF.

Another factor which is a candidate mediator for the pro-fibrogenic effects of hypoxia on PTE is PDGF [27,31]. PDGF is a multifunctional growth factor with effects on proliferation, chemotaxis, vasoconstriction and ECM regulation [43]. Three isoforms have been identified to date: PDGF-AA, PDGF-AB and PDGF- BB plus two PDGF receptor subunits denoted α and β, which can form three heterodimers, αα, αβ or ββ, depending on the PDGF isoform present [43]. PDGF-BB can bind to all three receptor combinations, PDGF-AB binds to either αα or αβ while PDGF-AA binds only to αα receptors [43]. PTE secrete both the A and B chains of PDGF and increased expression of mRNAs for PDGF-B chain and for the β receptor-subunit have been reported in the tubules of rats subjected to 5/6 nephrectomy [254]. In culture, mRNA levels for PDGF B-chain in human PTE are increased in cells derived from fibrotic kidneys [37] and increased tubulo-interstitial expression of PDGF and the β receptor has been observed in TIF; with a pronounced increase in expression in atrophic tubules [255].

PDGF is a potent mitogen for fibroblasts; *in vitro* experiments have demonstrated that PDGF released by inner medullary collecting duct epithelial cells induces proliferation in papillary fibroblasts which is blocked by the addition of PDGF anti-serum, demonstrating a paracrine mechanism for PDGF in these two cell types

[256]. Recent experiments have shown that a similar paracrine mechanism exists between epithelial and fibroblastic cells in human kidney cortex; PTE grown in co-culture with cortical fibroblasts release PDGF which stimulates fibroblast proliferation [46]. In addition to interstitial cell proliferation, treatment of rats with recombinant PDGF-BB also induces the appearance of interstitial myofibroblasts and subsequent interstitial fibrosis [257]. There is little information regarding the effects of PDGF on PTE matrix metabolism, however *in vitro* studies have demonstrated that CM from PTE induces collagen synthesis and modulates MMP activity in interstital fibroblasts and these effects are inhibited by the addition of anti-PDGF antibody [46].

As previously mentioned, PDGF may also be a likely candidate mediator for the hypoxia-induced changes in PTE ECM as this growth factor can be induced by O_2 depletion in a number of cell types [123]. The effect of hypoxia on the PDGF-A and -B chains is cell-type specific [123]; for example, the hepatic carcinoma cell lines, Hep3B and HepG2, both upregulate PDGF A and B mRNA chains when exposed to hypoxia, with PDGF-B more strongly induced than PDGF-A in HepG2 [123]. In addition, hypoxic HUVEC have increased PDGF-B chain [258] while in BeWo cells, hypoxia induces only PDGF-A mRNA [123]. The effects of hypoxia on PDGF expression in PTE have not been investigated.

Previous studies in our laboratory have suggested that ET-1 may also be a fibrogenic factor in the kidney [62,63,65]. The endothelins are a family of peptides with potent vasoactive and mitogenic actions; three members exist ET-1, ET-2 and ET-3 and to date, two receptors have been identified: ET_A and ET_B. ET_A binds with order of affinity ET-1=ET-2>ET-3 whilst the ET_B receptor binds all isoforms with equal affinity [64]. All three endothelin isoforms and both receptors are expressed in the kidney, however distribution is both cell-type and species-dependent [64].

In vivo, proximal tubules are known to secrete ET-1 [259] and *in vitro* human PTE express ET-1 and ET-2 mRNA and synthesise both ET-1 and ET-2 under basal conditions [62]. PTE express mRNA for both ET receptors but expression of ET_B is greater than that of ET_A [62]. As the renal microcirculation is particularly sensitive to the vasoconstrictor action of ET-1, most studies on the role of ET in the kidney have focused on this isoform [64]. Increased ET-1 peptide has been reported in both *in vitro* and *in vivo* models of chronic renal failure such as chronic proteinuria and the remnant kidney model [260,261]. In addition, exposure of PTE to albumin in an *in vitro* model of protein-overload, induces a dose-dependent increase in ET-1 synthesis [99]. ET-1 is known to be upregulated in PTE following ischaemic acute renal failure and this is associated with regeneration of surviving tubules after an ischaemic insult [259]. *In vitro* studies in our laboratory have demonstrated that, hypoxia (1% O_2 for 48hrs) induces ET-1 mRNA and protein secretion in human PTE [62]. Secreted ET-1 acting via ET_B receptors induces PTE proliferation and ligand/receptor binding itself can regulate ET-1 synthesis [62]. Hypoxia-induced auto-induction of ET-1 is thought to explain the long-lasting induction of ET-1 gene expression in the post-ischaemic kidney and may provide a mechanism for PTE regeneration in acute renal failure.

Another potent vasoactive factor implicated in fibrosis and induced by hypoxia in a variety of cell types is VEGF [230,231]. VEGF is a potent angiogenic factor in both physiological and pathological processes and is a powerful mitogen for endothelial cells *in vitro* [262]. To date, four VEGF isoforms have been identified: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$; the best characterised of these being $VEGF_{165}$ which is the isoform generally referred to as 'VEGF' [262,263]. Two receptors have been identified: KDR/*flk-1* and *flt-2* with potentially two more molecules *flt-3* and *flt-4* serving as receptors, although these have not yet been fully characterised [263]. There is little

information regarding VEGF expression in the kidney, however studies have shown that VEGF mRNA and protein levels are increased in human kidneys subjected to an ischaemic insult [230]. The effects of hypoxia on PTE *in vitro* have not been investigated, but VEGF is known to be upregulated in both primary cultures of human pigment epithelial cells, dermal fibroblasts and HUVEC and in the cell lines Hep3B and HeLa [161,264]. Although the effects of VEGF on cell matrix metabolism have not been fully investigated in detail, one report describes an upregulation of interstitial collagenase (MMP-1) in human endothelial cells [265], suggesting that VEGF may also function as a regulator of matrix metabolism.

The data in *Chapter 3* demonstrate that hypoxia can alter ECM metabolism in PTE. The studies in this chapter aim to determine whether hypoxia elicits these effects by autocrine actions of a hypoxia-induced factor.

4.2 Aims

- To investigate the role of TGF-β1 as a candidate mediator for the hypoxia-induced changes in PTE.
- If TGF-β1 is not the mediator, to determine whether a different soluble, secreted factor is responsible for the hypoxia-induced changes in ECM metabolism.

4.3 Methods

4.3.1. Bioassay for TGF-β

The CCL64 mink lung epithelial cell line (ECACC, Porton Down, Wilts, UK) bioassay [266] was used to measure levels of latent/active TGF-β (all isoforms) in PTE-CM.

CCL64 are growth inhibited by TGF-β, hence ^{3}H-TdR incorporation in response to TGF-β 1 standards can be used to quantify TGF-β in CM. Briefly, CCL64 were seeded at a density of 5x10^{3}/well in DMEM:F12, (1:1, Gibco-BRL) containing 10% FCS and incubated for 24hrs, the serum concentration was then reduced to 1% for a further 24hrs. Latent TGF-β was activated by heating CM samples at 80°C for 10mins. Samples were allowed to cool prior to addition to CCL64 cells. Activated or non-activated CM was diluted 1:1 with normal medium and added to each well for 24hrs. Cells were labelled with 1μCi ^{3}H-TdR /well (Amersham) for 5hrs prior to harvest, after which the cells were washed twice with PBS to remove serum proteins. Protein and DNA were precipitated with cold 10% TCA, solubilised and assayed as in the proliferation assay (*Chapter 2:2.3.2*). Concentrations of TGF-β in CM were measured by comparison to a standard curve of TGF-β1 (0-10ng/ml; Genzyme Diagnostics, West Malling, Kent, UK) prepared in PTE quiescence medium. Data are expressed as ng/ml active or total TGF-β in CM from PTE exposed to 24hrs hypoxia or after 24hrs re-oxygenation with respect to the relevant controls (*Fig. 4.2*).

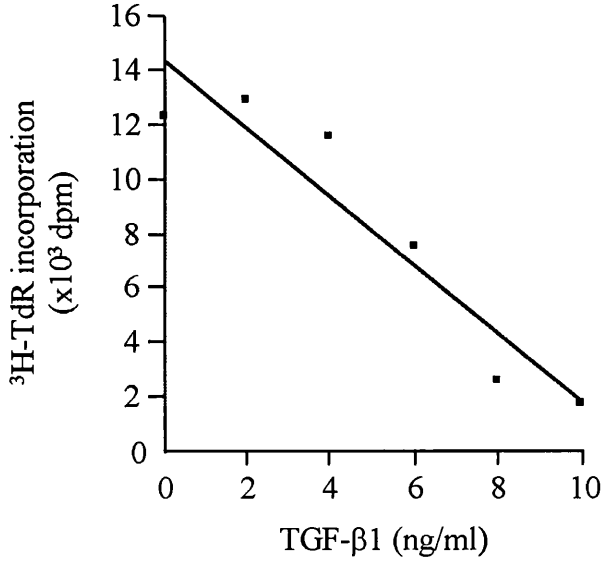

Fig. 4.2 ***^{3}H-TdR incorporation of CCL64 cells in response to TGF-β1.*** TGF-β1 (0-10ng/ml) was added to CCL64 for 24hrs and the cells labelled with 1μCi/well of ^{3}H-Tdr for 5hrs. Radioactivity was measured in the solubilised protein samples and 3H-TdR incorporation expressed as x10^{3} dpm/per well.

4.3.2. Effect of exogenous TGF-β1 on PTE

TGF-β_1 (2.5ng/ml or 10ng/ml; Genzyme Diagnostics) was added to quiescent PTE under normoxic conditions for 24hrs. Collagen I, Collagen IV, MMP-2 and TIMP-1 mRNA levels were measured by Northern Blot analysis and quantified by densitometry as described previously in *Chapter 2:2.3.9* . To investigate the effect of exogenous TGF-β1 on secreted MMP-2 activity, confluent, quiescent PTE were treated with 0, 2, 4, 6, 8, 10 ng/ml TGF-β1 for 24hrs under normoxic conditions. CM was collected, clarified and analysed by gelatin-substrate gel zymography (as described in *Chapter 3: 3.3.3*). Data are expressed as relative MMP-2 activity, calculated as fold over normoxia control (assigned a value of 1).

4.3.3. Neutralisation of TGF-β1 activity

To determine whether the effects of hypoxia on PTE are mediated by autocrine TGF-β1, anti-TGF-β1 antibody (chicken IgG: 1.2μg/ml or 10μg/ml; R&D Systems Europe Ltd., Abingdon, Oxon, UK) was added to cells immediately before hypoxia and at the beginning of the re-oxygenation period. To establish the specificity of the blocking effect, non-immune serum IgG (Sigma), at the same concentrations as the neutralising TGF-β1 antibody, was added to parallel cultures. The efficacy of the anti-TGF-β1 antibody was tested by simultaneous addition of antibody and exogenous TGF-β1 (10ng/ml; Genzyme Diagnostics) to PTE under normoxic conditions. MMP-2 activity was assessed by quantitation of gelatin-substrate gel zymography as described previously (*Chapter 3:3.3.3*) and the data presented as relative MMP-2 activity, calculated as fold over relative control (assigned a value of 1). Levels of mRNA expression of MMP-2, TIMP-1, Collagen I and Collagen IV in the presence and absence of exogenous TGF-β1, were measured by Northern Blot analysis and quantified as described previously (*Chapter 2:2.3.9*).

4.3.4. Effect of VEGF, PDGF and ET-1 on MMPs in PTE

The effect of human recombinant VEGF (gift from Dr. Ian Zachary, Dept. Medicine, RFUCMS, London, UK) , human recombinant PDGF-BB (Sigma) and ET-1 (a gift from Dr. Steve Mutsaers, Centre for Cardiopulmonary Research, RFUCMS, London, UK) on MMP levels were examined. VEGF (0, 2.5, 5, 10ng/ml), PDGF-BB (0, 2, 4, 6, 8, 10ng/ml) and ET-1(0, 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, 10^{-14} M) were added to confluent, quiescent PTE for 24hrs. The CM was collected, clarified by centrifugation at 2000xg for 15mins at 4°C and stored at -80° prior to zymography.

4.3.5. Effect of hypoxia on PDGF and VEGF mRNA expression in PTE

Northern analysis was performed as described in *Chapter 2: 2.3.9.* cDNA for human VEGF was a gift from Dr. Ian Zachary and cDNA for human PDGF (clone pSM-1) was obtained from ATCC.

4.3.6. Effect of hypoxic PTE-CM on Collagen I mRNA expression in naive PTE

CM collected from confluent, quiescent PTE exposed to either 24hrs hypoxia or normoxia was added to naive, confluent, quiescent PTE for 24hrs. The cells were harvested for RNA extraction and Northern Blot analysis of collagen α1(I) gene expression.

4.4 Results

4.4.1. Effect of hypoxia on TGF-β production

As measured by the CCL64 bioassy, hypoxia significantly increased the amount of active TGF-β (160% increase, $P<0.05$); 1ng/ml in control vs. 2.6ng/ml after 24hrs of hypoxia (*Fig.4.3.A*). Levels of total TGF-β in CM were also significantly increased following 24hrs hypoxia: 9.5ng/ml vs. 3.0ng/ml in control cultures ($P<0.05$, *Fig.4.3.B*).

Assay of CM from cells after re-oxygenation showed a decrease in both active TGF-β (1.2ng/ml vs. 4.0ng/ml in control) and total TGF-β (6.5ng/ml vs. 8.0ng/ml in control) suggesting that the increase induced by this degree of hypoxia is transient.

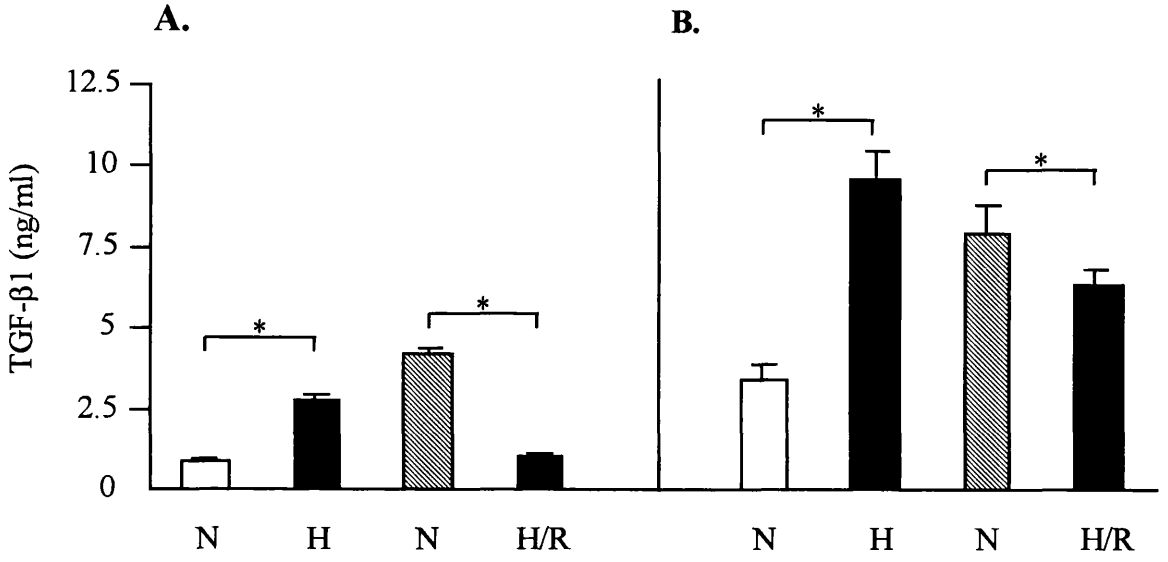

Fig. 4.3 ***Effect of hypoxia on levels of*** **(A)** ***active and*** **(B)** ***total TGF-β in PTE-CM.*** CM from control cells maintained for 24hrs in normoxia **(N)**; CM from control cells maintained for 24hrs in hypoxia **(H)**; CM collected from cells exposed to normoxia for 48hrs **(C)**; CM from PTE exposed to 24hrs hypoxia followed by 24hrs normoxia **(H/R)**. TGF-β concentrations in CM were calculated from a TGF-β1 standard curve (*Fig.4.2*). Data shown are a representative experiment of 3 repeats where all results were significant with a sample number of n=6 wells, $*P<0.05$, unpaired Student's T-Test.

4.4.2 Effect of exogenous TGF-β1 on MMP-2 mRNA and activity, TIMP-1, Collagen I and Collagen IV mRNA expression

Since TGF-β1 is regulates matrix synthesis and turnover in a number of cell types [204] and is known to be produced by PTE, experiments focussed on determining whether this TGF-β isoform might be the mediator of the effects of hypoxia on PTE matrix metabolism. PTE were treated with 0-10ng/ml TGF-β1 for 24hrs under normoxic

conditions, CM was collected for zymography and cells harvested for RNA extraction. To examine the effect of exogenous TGF-β1 on mRNA expression of MMP-2, TIMP-1, collagen I and collagen IV, two concentrations of TGF-β1 were tested: 2.6ng/ml; equivalent to the concentration of active cytokine produced by hypoxia-induced PTE, as calculated from the bioassay data (*Fig.4.3.A*) and 10ng/ml; equivalent to total TGF-β produced by PTE in hypoxia (*Fig.4.3.B*).

TGF-β1 (2.5ng/ml) stimulated MMP-2, TIMP-1, collagen I and collagen IV mRNA levels (data not shown). Levels were further increased by 10ng/ml TGF-β1, demonstrating a dose-dependent response to TGF-β1, (*Fig.4.3.A*). MMP-2 activity also increased in response to TGF-β1 (0-10ng/ml) in a dose-dependent manner (*Fig.4.3.B*).

4.4.3 Effects of anti-TGF-β1 antibody on PTE response to hypoxia

To examine whether the effects of hypoxia on PTE matrix metabolism are blocked by inhibition of TGF-β1, PTE were exposed to hypoxia in the presence of two concentrations of neutralising anti-TGF-β1 antibody: 1.2μg/ml, sufficient antibody to block all of the active TGF-β produced by the cells after 24hrs hypoxia (2.6ng/ml, calculated from TGF-β bioassay data (*Fig.4.3.A*)) and 10μg/ml, sufficient to neutralise total TGF-β produced by PTE under hypoxic conditions (active plus latent, 9.5ng/ml (*Fig.4.3.B*)).

In PTE treated with TGF-β1 (10ng/ml), 1.2μg/ml anti-TGF-β1 antibody blocked the TGF-β1-induced increase in MMP-2 activity (by 27%), MMP-2 mRNA (by 80%) collagen IV mRNA (by 48%), collagen I mRNA (by 56%) and TIMP-1 mRNA (by 62%) (data not shown). The higher concentration of antibody completely blocked MMP-2 activity and further decreased MMP-2 and collagen I mRNA expression. The specificity of the antibody was confirmed by the addition of non-immune IgG at

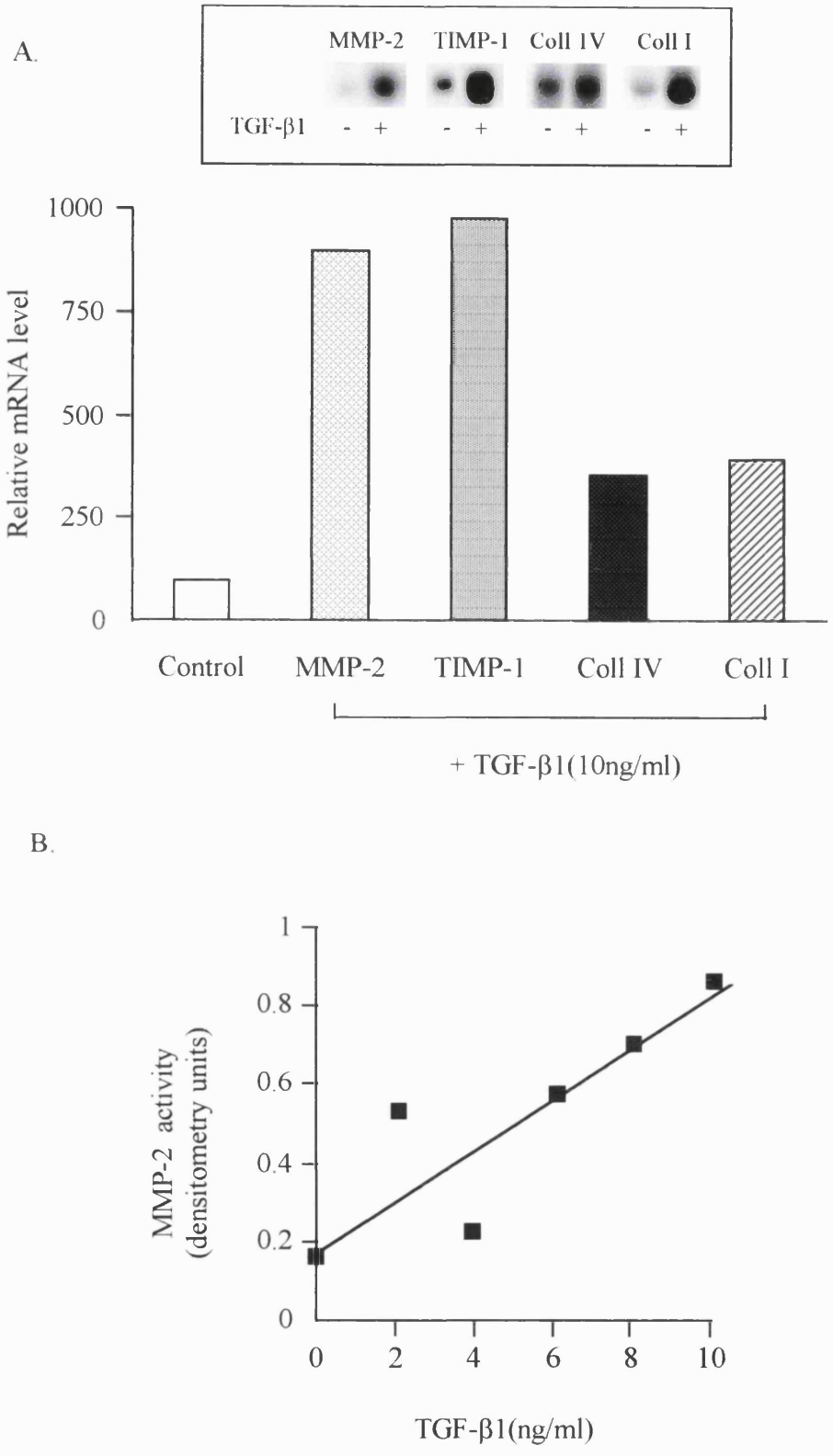

Fig. 4.4.A. ***Northern Blot analysis of MMP-2, TIMP-1, Collagen I, Collagen IV mRNA levels in normoxic PTE on addition of exogenous TGF-β1*** RNA was extracted from quiescent PTE maintained in normoxia for 24hrs (**Control**); normoxia for 24hrs in the presence of exogenous TGF-β1(10ng/ml) (+TGF-β1). The data show a representative experiment of 2 repeats. **Insert:** representative autoradiograms **B.** ***Effect of exogenous TGF-β_1 on MMP-2 activity in normoxic PTE.*** Confluent, quiescent PTE were treated with 0-10ng/ml TGF-β_1 for 24hrs. Gelatin-gel zymography was performed on CM and MMP-2 activity quantified by densitometry as described in *Chapter 2:2.3.9.*The figure shows a representative experiment of 2 repeats.

concentrations equivalent to the neutralising antibody, non-immune IgG had no effect on TGF-β1-induced increases in MMP-2 expression and collagen I mRNA levels (data not shown). Although the anti-TGF-β1 antibody had some small effect, neither concentration eliminated the hypoxia-induced decrease in MMP-2 activity (*Fig.4.5*).

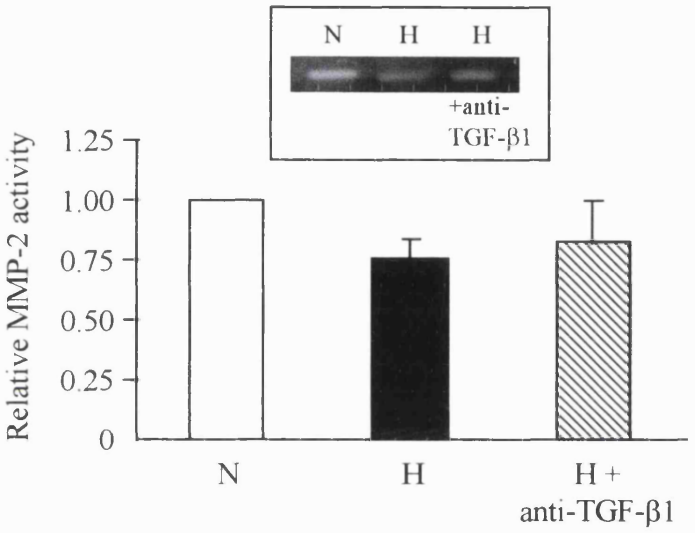

Fig. 4.5 ***Effect of anti-TGF-β1 antibody on MMP-2 activity in PTE exposed to 24hrs hypoxia.*** CM was collected from PTE exposed to 24hrs normoxia (N); 24hrs hypoxia (H) or 24hrs hypoxia in the presence of anti-TGF-β1 antibody (H+anti-TGF-β1). Gelatin-gel zymography was performed and quantified by densitometry. % change in MMP-2 activity in CM from treated PTE was calculated from arbitrary densitometry units with the relevant controls assigned the arbitrary value of 1. Data shown are the mean of 4 experiments ±SEM. **Insert:** Representative zymogram.

Similarly, the anti-TGF-β1 antibody had no effect on the hypoxia-induced increase in collagen I mRNA expression (*Fig.4.6*). Collagen IV, MMP-2 and TIMP-1 mRNA levels were also unaffected. The control IgG had no effect on hypoxia-induced changes.

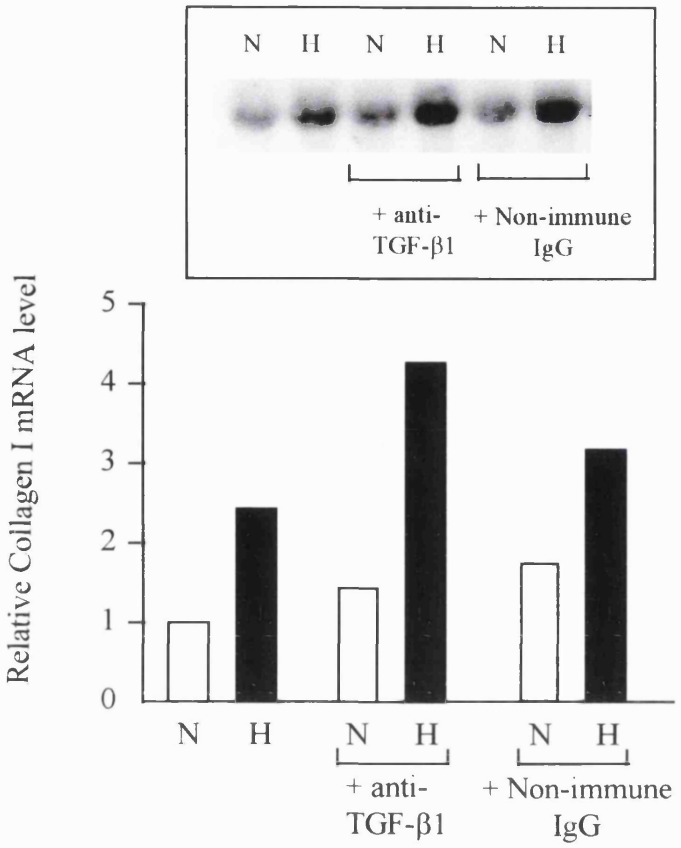

Fig. 4.6 ***Effect of anti-TGF-β1 antibody on collagen I mRNA*** **in** ***PTE exposed to 24hrs hypoxia compared to the addition of non-immume serum IgG.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21% O_2 (**N**); 24hrs hypoxia 1%O_2 (**H**); exposed to normoxia or hypoxia in the presence of 1.2μg/ml anti-TGF-β_1 (**+ anti-TGF-β1**); exposed to normoxia or hypoxia in the presence of 1.2μg/ml non-immune serum IgG (**+ non-immune serum IgG**). Autoradiograms were scanned and quantitated as described in *Chapter 2:2.2.9*. Fold increase over control in collagen I mRNA levels were calculated from densitometry units of the relevant controls, assigned a value of 1. The data show a representative experiment. **Insert**: representative autoradiogram.

4.4.4 Effect of hypoxia on PDGFand VEGF mRNA expression in PTE

As TGF-β1 did not appear to be the mediator of the hypoxia-induced changes, 2 other factors were investigated as potential effectors, PDGF-BB and VEGF. By Northern blot analysis PDGF-BB mRNA levels were increased 2.8 fold over control after 24hrs hypoxia (*Fig. 4.7*).

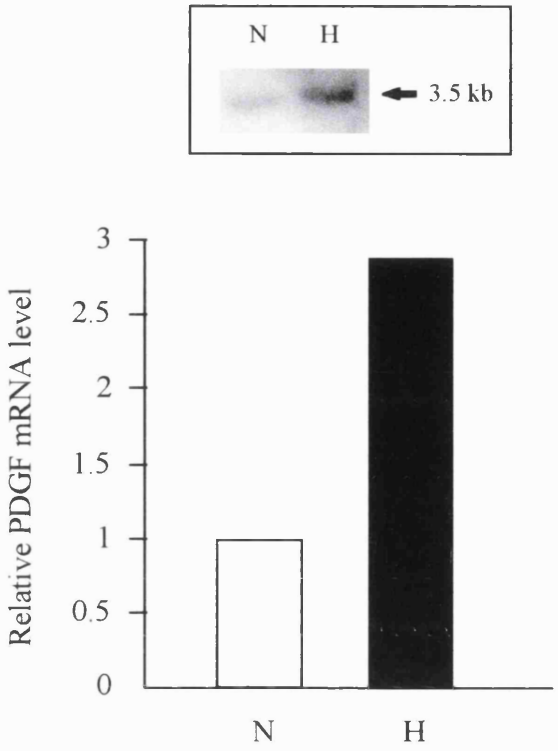

Fig. 4.7 ***Northern blot analysis of PDGF-BB mRNA in response to hypoxia.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21% O_2 (**N**); 24hrs hypoxia 1% O_2 (**H**). Autoradiograms were scanned and quantified by densitometry. Values were corrected for variations in RNA loading by comparison to densitometric values of ethidium bromide-stained rRNA. Fold change was calculated from densitometry units with controls assigned the value of 1. Data shown are a representative experiment of 3 repeats. **Insert:** representative autoradiogram.

VEGF mRNA levels were also markedly induced after 24hrs hypoxia (2.8 fold increase) but then rapidly decreased to below control level on return to normoxia. (*Fig.4.8*).

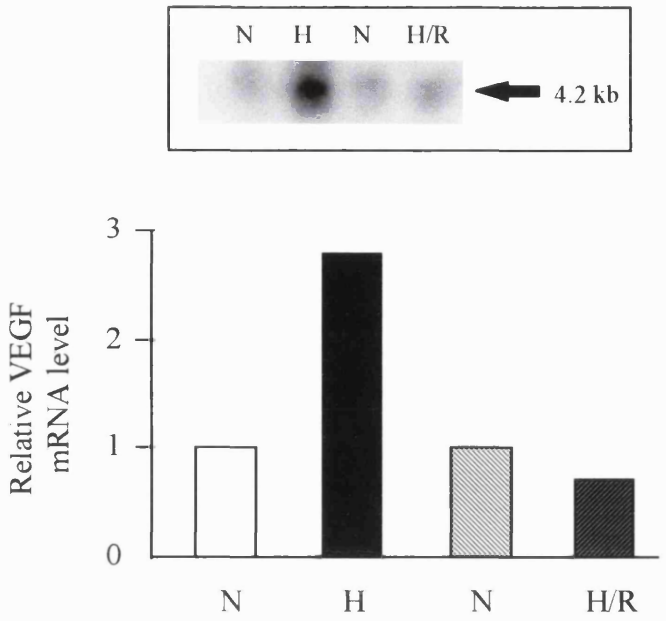

Fig.4.8. ***Northern blot analysis of VEGF mRNA.*** Total RNA was extracted from PTE exposed to 24hrs normoxia 21% O_2 (**N**); 24hrs hypoxia 1% O_2 (**H**); exposure to 48hrs normoxia (**N**); 24hrs re-oxygenation (**H/R**). Autoradiograms were scanned and quantified by densitometry. Values were corrected for variations in RNA loading by comparison to densitometric values of ethidium bromide-stained rRNA. Fold over control changes were calculated from densitometry units with controls assigned the value of 1. Data shown are a representative experiment of 3 repeats. **Insert**: representative autoradiogram.

4.4.5 Effect of exogenous growth factors on MMP-2 activity

To determine whether exogenous PDGF-BB or VEGF can mimic the effects of hypoxia on MMP-2 action, these growth factors were added to confluent, quiescent PTE and MMP-2 activity measured by zymography (*Fig.4.9*). In addition, ET-1 was tested since previous studies showed that ET-1 is up-regulated in human PTE exposed to 48hrs hypoxia [62].

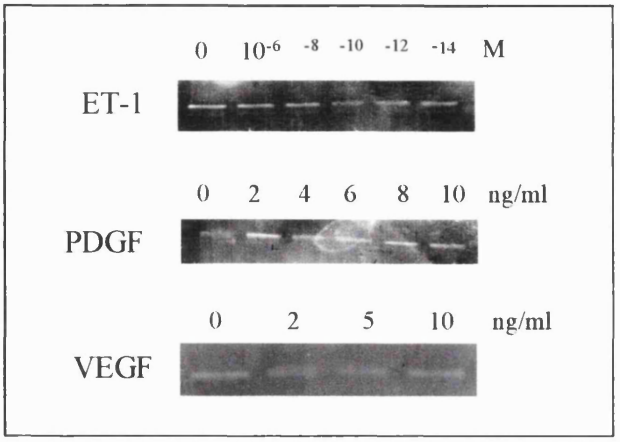

Fig. 4.9. ***Effect of exogenous VEGF, PDGF and ET-1 on MMP-2 activity in PTE.*** VEGF (human, recombinant, 0,2.5,5,10ng/ml), PDGF-BB (human, recombinant, 0,2,4,6,8,10ng/ml) and ET-1 ($0,10^{-6},10^{-8},10^{-10},10^{-12},10^{-14}$ M) were added to confluent, quiescent PTE for 24hrs. The medium was collected, clarified by centrifugation and zymography performed on samples containing equal protein. The photographs show zymography gels with cleared zones representing 72kD MMP-2 activity. Representative gels are shown.

None of the 3 growth factors tested had any effect on secreted MMP-2 activity in PTE under normoxic conditions (*Fig. 4.9*).

4.4.6 Effect of hypoxic PTE-CM on collagen I mRNA levels in naive PTE

Since none of the likely factors appeared to mimic the effects of hypoxia on PTE matrix production and turnover, conditioned medium transfer experiments were performed to establish whether there is a secreted soluble factor responsible for inducing the effects of hypoxia. Collagen I mRNA levels were examined after 24hrs incubation with CM collected from PTE subjected to hypoxia. As shown in *Fig.4.10,* CM from hypoxic PTE had no effect on collagen I mRNA levels.

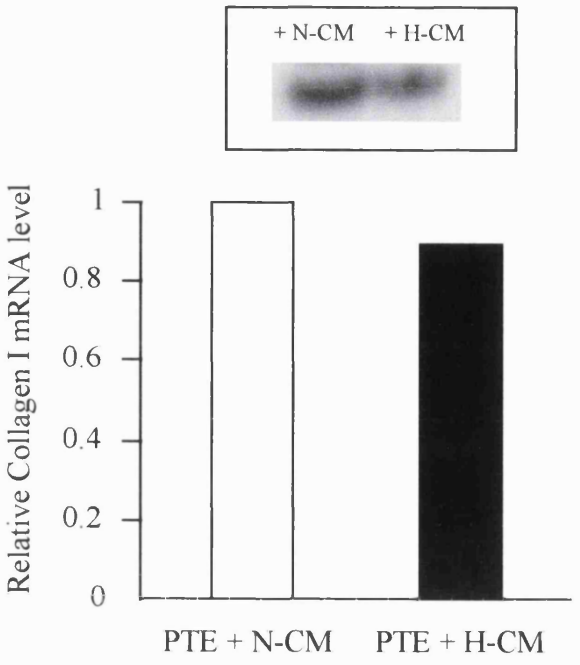

Fig. 4.10 ***Northern blot analysis of collagen I mRNA levels in naive PTE exposed to conditioned media collected from hypoxic PTE.*** CM was collected from PTE exposed to 24hrs normoxia 21% O_2 (**N-CM**) or 24hrs hypoxia 1% O_2 (**H-CM**) clarified by centrifugation, corrected for equal protein and added to naive PTE for 24hrs. Collagen I mRNA expression was measured by Northern blot analysis. Autoradiograms were scanned and quantified by densitometry as described in Chapter 2:2.3.9. Relative mRNA levels were calculated as fold change with respect to the control value (assigned value of 1). The data presented are a representative experiment of 2 repeats. **Insert** : representative autoradiogram.

4.4 Discussion

The data in *Chapter 3* showed that hypoxia is a fibrogenic stimulus for PTE *in vitro*. A number of growth factors are known to be induced by hypoxia, suggesting that the effects of O_2 depletion on PTE matrix metabolism might be mediated by the autocrine action of a hypoxia-induced growth factor. TGF-β1 is the most potent fibrogenic cytokine in the kidney [56] and expression of the factor by PTE is elevated in a number of TIF animal models [205]. Increased TGF-β mRNA and peptide levels are reported in proximal tubules in a rat model of acute renal failure, however, this is post-ischaemia after anoxic rather than hypoxic injury [267]. TGF-β1 is induced by hypoxia in a number of different cell types in culture including mesangial cells [163], dermal

fibroblasts [164], and BeWo cells [123] however there is currently no information regarding the effects of hypoxia on TGF-β levels in PTE. Initial experiments therefore focussed on TGF-β1 as a potential mediator of the hypoxia-induced effects on PTE matrix metabolism.

Levels of secreted TGF-β (all isoforms) were measured in PTE CM using the CCL64 mink lung epithelial cell bioassay. Unlike measurement of TGF-β protein levels in CM by Western analysis or ELISA, the CCL64 assay allows measurement of latent and biologically active TGF-β protein. Normoxic PTE-CM contained both active and latent forms of TGF-β protein in a ratio of approximately 1:3. Exposure of PTE to hypoxia (24hrs, 1% O_2) significantly stimulated the secretion of both active (3.2 fold) and latent (2.6 fold) TGF-β and decreased the ratio of active vs latent forms (1:2.4). This demonstrates for the first time that hypoxia not only stimulates the production of TGF-β in PTE but also increases bioavailability, perhaps by inducing enzymes which cleave the latent TGF-β isoform or by increased stability of the active form of growth factor. After 24hrs re-oxygenation, both active and latent TGF-β levels were significantly decreased with respect to normoxic controls. The ratio of active to latent protein was increased from 1:2 in controls compared to 1:5 in re-oxygenated cells. The decline in TGF-β levels during re-oxygenation suggests that either hypoxia *per se* is required in order to maintain high levels of TGF-β or that once secretion is induced, a cascade of events is initiated which does not require sustained TGF-β production.

The CCL64 bioassay used to quantitate TGF-β does not differentiate between the different isoforms but since it is known that tubular epithelial cells produce mainly TGF-β1, it is likely that this is the major isoform secreted and upregulated by hypoxia. Although it is unclear whether the hypoxia-induced increase in TGF-β protein is due to increased gene transcription, increased mRNA stability or post-transcriptional events,

hypoxia-induced changes in TGF-β mRNA levels have been demonstrated in several cell types. In human dermal fibroblasts, TGF-β1 peptide levels are elevated after 24-72hrs hypoxia whilst mRNA levels are not increased until 72hrs of hypoxia [164]. In this cell type, the increased TGF-β1 mRNA levels are due to increased gene transcription rather than increased mRNA stability [164]. Although a HRE (discussed further in *Chapter 5*) has not yet been identified for the TGF-β1 gene, the presence of a HRE has been suggested since the TGF-β superfamily of proteins contain a cysteine knot which is also present in other hypoxia-inducible growth factors such as PDGF [123,268]. One other possibility is that the TGF-β1 gene is not directly induced by hypoxia but is the target of a different hypoxia-inducible growth factor. It has been demonstrated in an in vitro model of diabetic nephropathy that PDGF can induce TGF-β1 protein production in human PTE [233] and that the PDGF gene itself is upregulated by hypoxia in some cell types [123], suggesting another possible mechanism for the observed hypoxia-induced increase in TGF-β protein levels.

In order to establish whether TGF-β1 was responsible for the effects of hypoxia on PTE matrix metabolism, a neutralising antibody to this factor (at sufficient concentration to block active TGF-β and total TGF-β) was added to cells during exposure to hypoxia (24hrs). This antibody did not block the hypoxia-induced changes in any of the parameters, suggesting that TGF-β1 is not responsible for the observed effects of hypoxia on PTE. Furthermore, the effects observed when exogenous TGF-β1 was added to PTE differ from the changes induced by hypoxia (*Table 4.3*), providing further evidence against a role for TGF-β1.

	Hypoxia	TGF-β1
Collagen production	↑	ND
Collagen I mRNA	↑	↑
Collagen IV mRNA	↓	↑
MMP-2 mRNA	NC	↑
activity	↓	↑
TIMP-1 mRNA	(↑)	↑
protein	↑	ND
TIMP-2 mRNA	↑	ND

Table 4.3 ***Comparison of the effects of hypoxia and exogenous TGF-β1*** . NC= No change, ND= No data, (↑) = change with re-oxygenation

The role of TGF-β1 as mediator of hypoxia-induced effects on ECM metabolism has also been investigated in other cell systems. In contrast to the data for PTE, the induction of collagen I by hypoxia in mesangial cells has been attributed to this factor; however TGF-β1 neutralising experiments were not conducted in this study [163]. Also, in contrast to the findings here, TGF-β1 is reported to be responsible for the increase in collagen I mRNA levels in dermal fibroblasts exposed to hypoxia; in this study, anti-TGF-β1 antibodies blocked the induction of collagen I [164]. It is therefore possible that the mechanisms which mediate collagen I induction by hypoxia are cell-type dependent as has been demonstrated for other hypoxia responses [123].

Since TGF-β1 does not appear to mediate the effects of hypoxia on PTE ECM metabolism, the importance of hypoxia-induced TGF-β1 protein levels therefore remains

unclear. One possibility is that TGF-β1 may be responsible for other aspects of the fibrogenic profile induced by hypoxia eg. the effect of hypoxia on integrin expression in PTE. Preliminary data has shown that PTE have elevated αvβ3 mRNA and protein levels in PTE in response to hypoxia, providing another mechanism by which hypoxia can alter ECM metabolism in PTE [228]. As mentioned previously, TGF-β1 upregulates αvβ3 expression in a human lung fibroblast cell line [250], raising the possibility that elevated TGF-β1 levels in response to hypoxia, may affect PTE αvβ3 integrin expression.

In addition to effects on ECM, TGF-β1 can also regulate cellular proliferation [60]; while hypoxia does not alter PTE proliferation *in vitro*, increased TGF-β1 production by PTE *in vivo* may act in a paracrine manner to alter the proliferation of neighbouring cell types. It has been demonstrated that TGF-β1 is a mitogenic factor for interstitial fibroblasts and one feature of TIF is increased proliferation of the cortical fibroblast population [269]. Furthermore, TGF-β1 is a differentiation factor and studies have shown that it can induce a myofibroblastic phenotype in interstitial fibroblasts [229]; one can therefore speculate that one role for the hypoxia-induced increase in TGF-β1 *in vivo* is as a paracrine effector.

Although the data reported here suggest that TGF-β1 is not the primary mediator of the pro-fibrogenic effects of hypoxia on PTE, the possibility that the changes observed in PTE ECM metabolism with hypoxia may have been due to TGF-β2, the other TGF-β isoform expressed by PTE, cannot be excluded since the measurement of secreted TGF-β includes all isoforms, while the neutralising antibody used blocks only the β1-isoform. Alternatively, another hypoxia-induced growth factor may be responsible for the observed effects. Candidate factors include PDGF, ET-1 and VEGF, all of which have been implicated in renal fibrosis [32,43]. PDGF is a hypoxia-inducible

growth factor [123] and a potent inducer of matrix protein gene expression in a number of different cell types [43]. In addition, studies have shown increased PDGF-BB expression in PTE cultured from fibrotic kidneys compared to cells from normal kidneys [37]. In view of this information and the knowledge that PTE are known to produce PDGF-BB, PDGF-BB was considered a possible mediator of the fibrogenic effects of hypoxia. PDGF-BB mRNA expression was induced by hypoxia in PTE, demonstrating for the first time that this growth factor is regulated by decreased O_2 in this cell type. This suggested that PDGF-BB may be the mediator of hypoxia-induced changes in matrix metabolism.

One other candidate factor was VEGF. Although VEGF is not generally considered to be a fibrogenic factor, it is known that the VEGF gene contains a hypoxia-responsive element [270] and VEGF is induced in a number of cell types exposed to decreased O_2 levels [161,264]. Furthermore, PTE VEGF mRNA expression is increased in *in vivo* models of fibrosis [230]. As with PDGF, VEGF mRNA expression was induced by hypoxia, a finding that has not previously been reported for PTE *in vitro*. This induction suggested that this growth factor may also have a role as a candidate mediator for the pro-fibrogenic effects of hypoxia on PTE. However, the addition of either exogenous PDGF-BB or VEGF failed to mimic the effect of hypoxia on MMP-2 activity. Furthermore ET-1, another possible candidate mediator, also had no effect on MMP-2 levels when added to PTE. These data suggest that these growth factors do not mediate the hypoxia-induced changes in ECM metabolism however it cannot be assumed that all of the effects on PTE ECM are mediated by the same factor and the possibility remains that PDGF, VEGF and ET-1 may mediate aspects of the response other than MMP-2 activity.

Although the data suggest that VEGF is not the mediator of at least one of the hypoxia-induced effects on PTE ECM, increased mRNA levels by PTE are in accordance with *in vivo* data in which there is high expression of VEGF mRNA in the proximal tubules of kidneys subjected to ischaemic injury [230]. VEGF is a potent permeabilizing factor [263] and one can speculate that its release by PTE subjected to hypoxia *in vivo*, would injure neighbouring capillaries, resulting in exacerbation of the ischaemic injury. If the hypoxia-induced changes in PTE demonstrated *in vitro* mimic those which occur *in vivo*, this would potentially lead to further deposition of ECM in the tubulo-interstitial compartment.

The studies outlined above suggest that none of the likely candidate factors mediated the effects of hypoxia on ECM production and turnover. Rather than continue to screen individual candidate factors, conditioned medium transfer was used to establish whether there is any soluble secreted factor(s) that induce the hypoxic phenotype in PTE. Measurement of MMP-2 activity as a representative hypoxia-induced parameter was not possible in this experiment (since hypoxic PTE-CM already contains MMP-2) and so collagen I mRNA expression was measured. Hypoxic PTE conditioned media had no effect on collagen I mRNA expression in naive cells, suggesting that the mechanism of induction of at least this aspect of the response is unlikely to be through the action of a stable, soluble, secreted factor.

4.6 Summary and conclusions

- These data suggest that TGF-β1 is not the primary mediator of the hypoxia-induced changes in ECM metabolism.
- Examination of other potential mediators: PDGF, VEGF and ET-1 demonstrated that these also are not responsible for the hypoxia-induced effects.
- Addition of conditioned medium from hypoxic PTE to naive PTE failed to mimic the effect of hypoxia on collagen I mRNA suggesting a direct effect of hypoxia on this gene.

CHAPTER 5

Regulation of the Collagen α1(I) Promoter by Hypoxia

5.1 Background

The data presented in *Chapter 4* demonstrate that the hypoxia-induced increase in collagen α1(I) mRNA is not mediated by a secreted autocrine factor. This raised the possibility that induction occurs by the direct action of hypoxia on expression of the collagen α1(I) gene. As mentioned in *Chapter 1*, a large number of genes are regulated by hypoxia including those encoding growth factors, glycolytic enzymes and vasoactive factors (see *Table 1.3*), however there is currently no information regarding the direct effects of hypoxia on matrix genes. In the few studies examining hypoxia-mediated changes in the ECM, the direct effects of hypoxia have not been considered and. only steady-state mRNA changes have been reported [163, 164, 210].

The finding that PTE exposed to hypoxia have increased levels of this atypical, interstitial collagen α1(I) was of particular interest; as increased PTE collagen I expression in response to hypoxia is in accordance with the phenotype observed both in *in vitro* and *in vivo* studies of tubulo-interstitial fibrosis. Collagen I expression appears within the PTE basement membrane in both animal models [50] and human TIF [51] and increased collagen I mRNA levels have been demonstrated in PTE cultures derived from fibrotic kidneys [50]. Since collagen I is a major interstitial collagen which accumulates in TIF [5, 26, 223], it was of interest to examine whether hypoxia can act directly on the collagen α1(I) gene. In addition, although PTE in culture have low basal expression of collagen I, this collagen sub-type is produced by mesenchymal cells [26,223]. Since, developmentally PTE are derived from the nephrogenic mesenchyme, hypoxia-induced

upregulation of collagen I may be indicative of a pathological transdifferentiation process [227].

As discussed in *Chapter 3*, collagen I is the product of two genes which encode the α1(I) and the α2(I) chains respectively [188]. Although located on different chromosomes, the two genes are co-ordinately regulated to form a heterotrimeric protein composed of two α1(I) and one α2(I) polypeptide chains expressed in a developmental and tissue specific manner [271,272]. The co-ordinated expression of the 2 collagen I chains is poorly understood but appears to occur at the transcriptional level; a recent study proposes that a polypyrimidine tract present in both the α1 and α2 promoters binds a Y-box protein, YB-1 which may be involved in co-ordinating transcription of the 2 genes [273].

Collagen production is controlled at multiple levels (*Chapter 3*), however the most important regulatory events are thought to occur at the level of gene transcription [274,275]. Collagen I genes are transcribed in many cell types and their regulation is accordingly complex [271]. Analysis of the collagen I genes by transient-transfection assays, *in vitro* transcription, and the generation of transgenic animals has revealed a large number of *cis*-acting regulatory elements situated both upstream and downstream of transcription initiation [271,272]. *Cis*-acting sequences bind specific nuclear transcription factors (*trans*-acting factors) to either positively or negatively influence transcription [271]. Whilst some of these elements may be present in both collagen I genes and appear to be conserved across species [188], others are unique to a particular chain and are species specific [276,277]. Detailed analysis of the promoter regions of both collagen α1(I) and α2(I) has revealed many *cis*-acting elements capable of directing transcription, although the two promoters themselves show little sequence homology [272].

Although numerous studies have examined collagen α1(I) gene regulation, a published diagram illustrating the important transcription factor binding-sites present on the gene promoter could not be located. Therefore, as a first step in looking at regulation of the collagen α1(I) promoter by hypoxia, *Figures 5.1.A* and *5.1.B* were compiled from a number of published sources. These figures indicate important transcription factor binding-sites present in the mouse and human collagen 5'-α1(I) promoter, spanning -2310/+115, since most studies have focussed on this region.

In both the human and mouse collagen α1(I) promoters, a number of important regulatory elements are situated in the proximal 300bp upstream of the start site [271]. Studies by Rippe et al demonstrated that a short segment of 5' promoter spanning -200 to +16, is sufficient for expression of the mouse gene whereas further more distal 5'sequences (up to -2.5kb) have a negative effect on transcription [282]. Similarly studies of the human collagen α1(I) promoter have shown that the region spanning -300 to +100 directs transcription of a reporter gene in murine and avian fibroblasts [281]. Examination of this region identified a -CCAAT- motif close to the transcription start site which binds the CCAAT box-binding factor (CBF) [281]. CBF is a ubiquitously expressed protein which is known to activate transcription of the collagen α1(I) gene [281]. Situated close to the CBF binding site are two regions where an NF-1-binding site is found to overlap with an Sp1-binding element [278]. NF-1 and Sp1 are ubiquitously expressed transcription factors and NF-1/Sp1 sites have been shown to be critical for high constitutive expression of the murine collagen α1(I) gene in mouse 3T3 fibroblasts [278]. A variety of experiments have demonstrated that overexpression of Sp1 inhibits mouse collagen α1(I) promoter activity in transiently-transfected 3T3 cells whilst overexpression of NF-1 stimulates promoter activity [278]. Since these sequences overlap, binding of one factor is thought to prevent binding of the second, thus steric

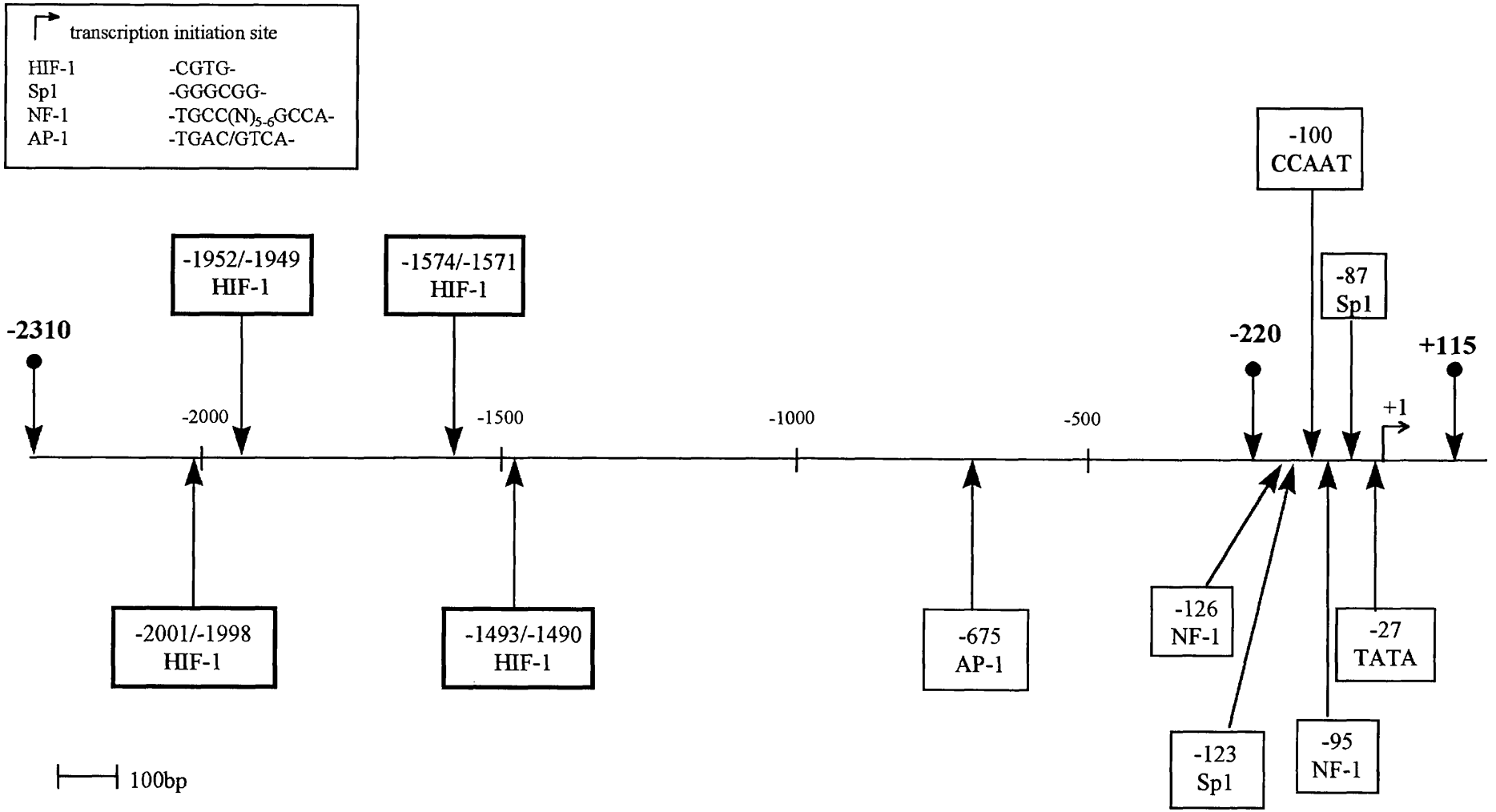

Fig. 5.1.A ***Schematic diagram of murine collagen α1(I) promoter -2310/+115.*** Diagram shows reported transcription factor binding sites and 4 HIF-1 binding sites -CGTG- as identified from collagen α1(I) promoter sequences [188, 278-280].

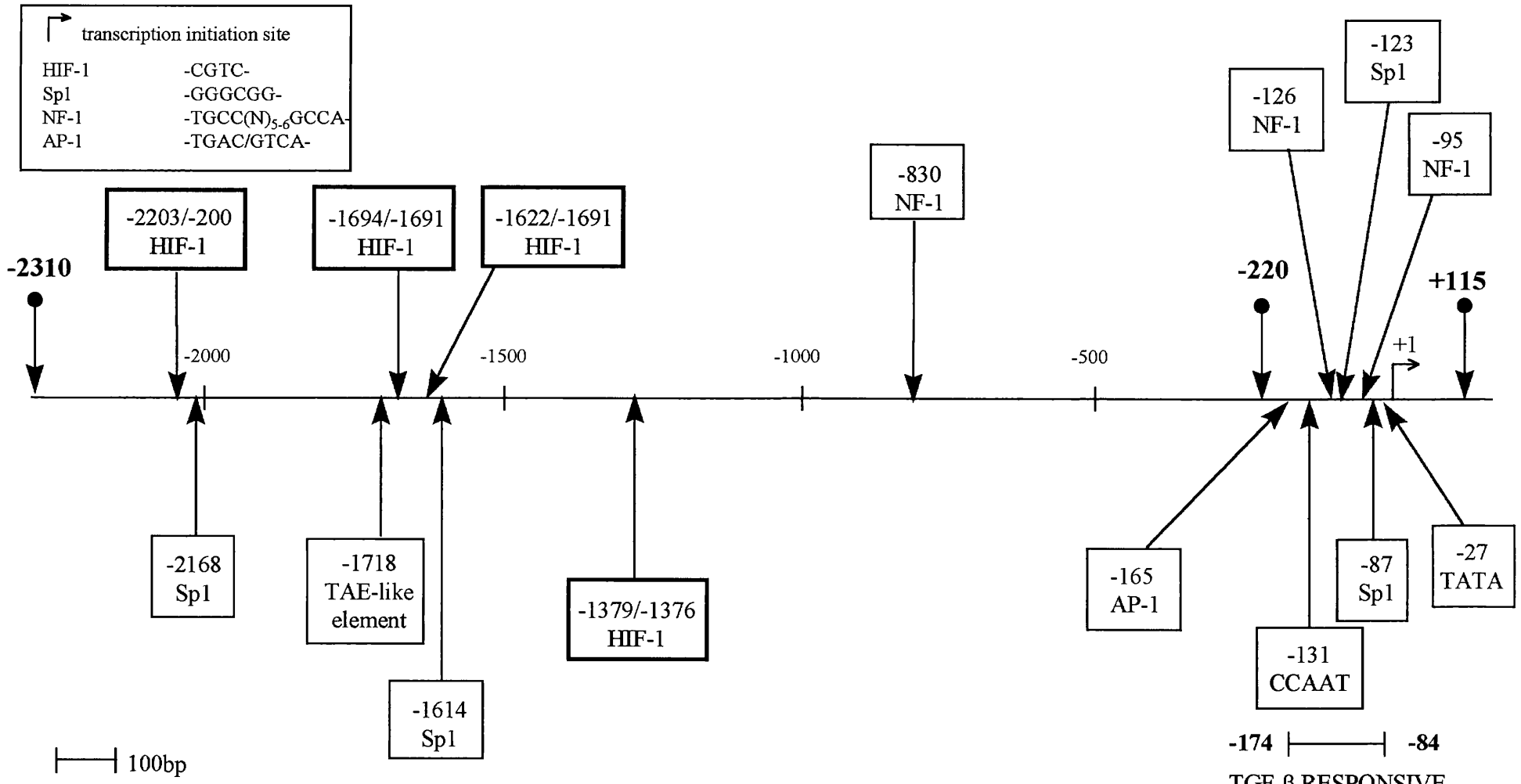

Fig. 5.1.B ***Schematic diagram of human collagen α1(I) promoter -2310/+115.*** Diagram shows reported transcription factor binding sites and 4 HIF-1 binding sites -CGTG- as identified from collagen α1(I) promoter sequences [188, 206, 278,281].

hindrance modulates promoter activity [278]. As the two NF-1/Sp1 binding regions are conserved within the human and mouse promoters it is likely that these regions have an important regulatory role in transcription of the collagen α1(I) gene [278].

In addition to NF-1 and Sp1, numerous AP-1 sites have been identified within the collagen α1(I) promoter [276,280]. The canonical AP-1 site was first described as a DNA-binding sequence in the enhancer of the Simian Virus 40 gene and subsequently found in a number of eukaryotic genes where it was shown to be activated through interaction with members of the *fos* and *jun* families of transcription factors [280]. An important AP-1 site has been identified in the mouse collagen α1(I) promoter; increased gene expression in the tight skin mouse, which develops fibrosis, is thought to result from lack of binding of the AP-1 transcription factor to a negative regulatory site situated at -675kb, which normally inhibits gene transcription [280].

The expression of collagen I is cell-type specific and regulatory elements have been described within the collagen α1(I) gene which ensure transcription within the appropriate cells [271,272]. Cell-specific expression is the result of DNA interaction with binding proteins that are expressed or active only in certain cells [274]. In addition, such factors as methylation status and chromatin conformation of the DNA limit the accessibility of *cis*-elements to *trans*-acting factors to ensure cell specificity [274]. In the mouse promoter, a 117bp segment (-1656/-1540), is the minimal sequence able to confer high level expression in osteoblasts of transgenic mice and this region binds an as yet unidentified nuclear protein, expressed only in osteoblasts [276]. In contrast, expression of the collagen α1(I) gene in skin fibroblasts requires -900bp of 5' promoter [276] and in hepatic stellate cells, regulatory sequences have been identified 440bp upstream of the transcription start site [277].

In addition to examining the elements involved in tissue specificity, a number of studies have focussed on the regulatory sequences responsible for cytokine modulation of collagen I [272,275]. TGF-β1 is known to be a potent stimulator of collagen I gene expression and the mechanisms of regulation have been studied in some detail [279,283]. A TGF-β activator element (TAE) has been described in the rat collagen α1(I) gene and is located 1.6kb upstream from the transcription initiation site [208]. This TAE consists of an AP-2 site within an NF-1-like binding motif: $TGCG(N)_5GCCAAG$ [208]. The proteins binding to this region have not been identified but appear to be distinct from NF-1 and AP-2 [208]. A similar TAE has been described in the human gene, located at around -1718bp, however transfection of 3T3 fibroblasts with a promoter construct containing a 5kb deletion (lacking the putative TAE) did not prevent TGF-β stimulation of promoter activity, suggesting that, in this cell type, the NF-1 like element is not required for TGF-β-induced activation of gene transcription [206]. In these studies, a separate region (-174/-84bp) was identified as being responsive to TGF-β1 but the *trans*-acting factors which bind to this have not yet been identified [206]. No TAE has yet been identified in the mouse collagen α1(I) gene however in the α2(I) gene, a region between -300bp and -350bp, which overlaps an NF-1-binding site, is essential for TGF-β1-stimulated transcription [207].

Transcriptional regulation of collagen gene expression is thought to be mediated not only by the promoter but through interaction between promoter sequences and *cis*-acting elements located both upstream and downstream of the transcription start site [272]. Several studies have suggested that the first intron of the collagen α1(I) gene is crucial for transcription however recently the role of this intron has become controversial [284]. The first intron contains both positive and negative regulatory sequences [188]; the best characterised of these being an AP-1 site situated between +494/+854 in the

human gene which can either positively or negatively influence transcription depending on the cell type [285]. Many of these experiments have involved the use of collagen gene constructs containing intronic sequences placed upstream of the promoter region in either a 5' or 3' orientation, in an attempt to enhance promoter activity. This has consequently led to conflicting data from transfection studies in different systems using different cell types [284]. A recent study by Hormudzi et al used a gene-targeting approach to generate transgenic mice with a mutated collagen α1(I) allele in which the first intron has been deleted [286]. The data demonstrated that, in skin, the intron appears to play no role in constitutive expression of collagen α1(I), whilst in the lungs of young transgenic mice, expression was approximately 75% of that in wild type mice and in the adult lung, less than 50% of control animals [286]. Thus, the first intron seems likely to play a tissue-specific and developmentally-regulated role in transcriptional regulation of the gene.

In addition to 5'flanking and intronic sequences, the 3' flanking region of the mouse collagen α1(I) gene is also involved in transcriptional regulation [272]. An E-box situated between +1899/+4597 contains sequences which bind the helix-loop-helix proteins USF-1 and USF-2 (upstream stimulatory factors) to stimulate transcription [287]. USF proteins are involved in the regulation of many genes including those induced in a tissue-specific and inducible manner; it is therefore likely that in addition to sites in the promoter, the 3' flanking region confers tissue-specificity to the collagen α1(I) gene [287]. Taken together, all of the studies described above demonstrate that the collagen α1(I) gene is regulated by a complex array of *cis*-acting elements in the 5'-flanking region, the promoter, the first intron and the 3'-flanking region. Ubiquitous and collagen-specific *trans*-acting factors act in concert to regulate collagen gene transcription and appropriate tissue specific expression.

Although the collagen α1(I) promoter has been studied extensively, the hypoxia-inducibility of the promoter and presence of specific hypoxia-response elements have not yet been investigated. As mentioned in *Chapter 1*, the first HRE was described within a 50bp region of the 3'flanking sequence of the EPO gene [132,144]. Under conditions of hypoxia, a specific core sequence, -CGTG-, was found to bind the heterodimeric transcription factor HIF-1 [149]. Since this initial finding, a more widespread O_2 -sensing mechanism has been discovered within non-EPO producing cells and a large number of hypoxia-inducible genes have been identified which require HIF-1 binding activity for O_2 -regulated transcription [150,151].

It is now recognised that HIF-1 cannot function in isolation; HIF-1 interacts with adjacent transcription factors and co-activating proteins to form multiprotein complexes [156,157]. In a recent model proposed for hypoxia-induced EPO expression, the complex includes HIF-1, hepatic nuclear factor-4 (HNF-4) and the co-factor p300 [157](*Fig. 5.2*).

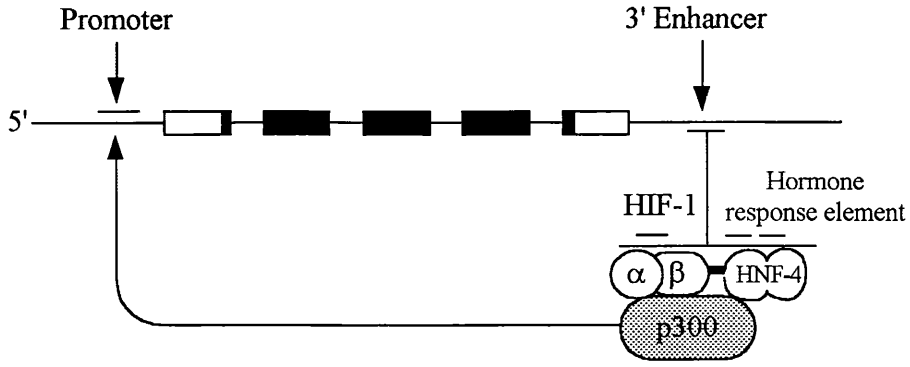

Fig. 5.2 ***Model of the multiprotein complex assembled on the 3' enhancer of the EPO gene in hypoxia*** (adapted from [288]).

Downstream of the HIF-1 binding site, the EPO 3' enhancer contains two tandem consensus steroid hormone response elements which constitutively bind HNF-4; an

interaction which is thought to contribute not only to the high level of EPO induction but also to its tissue-specificity [157]. During hypoxia, the two HIF-1 sub-units associate and bind to the HIF-1 site within the enhancer. HIF-1 binds specifically with HNF-4 and both transcription factors then associate with the transcriptional activator p300. This complex transduces a signal to the promoter and transcription is activated [157,288]. Similar hypoxia-induced multi-protein complexes have been proposed for other genes; with different accessory proteins associating with HIF-1 depending on the gene in question [157].

In addition to accessory sites, multiple HIF-1 binding regions in close vicinity are required for hypoxia-responsiveness of some genes, for example, in the transferrin promoter, a HRE has been identified which contains two HIF-1 binding sites in a region spanning 32bp [133]. Mutation of both of these regions is required in order to abolish the hypoxia response, demonstrating that more than one HIF-1 site may be required for hypoxia-induced transcription of some genes [133]. Although the presence of a HIF-1 binding site is often indicative of hypoxia-responsiveness, it does not guarantee HIF-1 binding and hypoxic activation [151]. Furthermore, it is known that other transcription factors can bind to the canonical 5'-CGTG-3'; in the EPO gene ATF-1 and CREB family members are capable of constitutively binding at the HIF-1 sites [157], although it is currently unclear if these factors are displaced by HIF-1 during hypoxia or whether they form part of a hypoxia-induced multiprotein complex, similar to that proposed in *Fig. 5.2*. A recent study investigating hypoxia-induced transcription of the LDH-A gene demonstrates that ATF-1/CREB associates with HIF-1 and p300 during hypoxia, providing evidence for the latter suggestion in this gene [157].

Since the discovery that HIF-1 activates EPO transcription in response to hypoxia, HIF-1 has been implicated in transcriptional activation of many different

hypoxia-regulated genes [151]. However, it is now recognised that HIF-1 is not the only transcription factor activated by hypoxia [154,155,158,159]. Recent work has identified another basic-helix-loop-helix transcription factor inducible by hypoxia which was initially thought to be expressed predominantly in endothelial cells but may have more widespread distribution. Endothelial PAS domain protein 1 (EPAS-1 also known as HIF-2α, MOP2, HLF, HRF) [154,155] is 48% homologous to HIF-1α, can dimerise with HIF-β and recognises the HIF-1 binding site [155]. The discovery of this factor raises the possibility that HIF-1 is part of a larger family of hypoxia-inducible transcription factors and other members may yet be identified. In addition, the finding that widely expressed transcription factors such as AP-1 are hypoxia-responsive suggests that alternative non-HIF-mediated hypoxia-responsive pathways may exist. AP-1 induction by hypoxia in HeLa cells is mediated by increased transcription of the *c-fos*, *jun-D* and *c-jun* genes in response to changes in cellular redox status, although the mechanism for AP-1 induction by ROS remains unclear [158]. One other ubiquitous transcription factor which may be activated by hypoxia is NF-κB, however it remains unclear if hypoxia *per se* is the activator or whether the induction is dependent on re-oxygenation [158,159]. In contrast to AP-1, NF-κB is predominantly regulated at the post-translational level [158]. This involves phosphorylation and subsequent degradation of an inhibitory sub-unit, IκB which retains NF-κB in a cytoplasmic complex and prevents its nuclear transport and DNA binding [158]. It is not currently understood how hypoxia/re-oxygenation mediates these post-translational events leading to activation of NF-κB.

As discussed in *Chapter 1*, HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits; while HIF-1α is unique, HIF-1β is identical to ARNT, the aryl hydrocarbon receptor involved in the xenobiotic response [152]. Regulation of HIF-1 by

hypoxia is complex and acts at both the transcriptional and post-transcriptional levels [152,289]. Measurement of basal levels of HIF-1 mRNA expression in a variety of cell lines has demonstrated that levels vary greatly between different cell types and generally when basal levels are high, there is little or no induction in response to hypoxia [290], while in cells with low basal expression, HIF-1 mRNAs are elevated in response to 1% O_2, typically peaking after 1-2 hours and declining to basal values by 4-8 hours [152]. Analysis of HIF-1 protein levels in Hep3B cells has demonstrated that under normoxic conditions, HIF-1β is detected in both nuclear and cytoplasmic extracts while HIF-1α is not detected in either of these compartments [152]. In these studies, when cells were transferred to 1% O_2, there was a dramatic increase in nuclear HIF-1α levels whilst HIF-1β was lost from the cytoplasm and accumulated in the nucleus [152]. On re-oxygenation after 4hrs of hypoxia, HIF-1β levels reappeared in the cytoplasm but HIF-1α levels were completely degraded, demonstrating a DNA-binding activity that is tightly regulated by O_2 tension [152]. Accumulation of HIF-1α protein in the nucleus was detected under conditions in which HIF-1α mRNA levels were induced [152] or remained constant [291] indicating that the regulation of steady-state HIF-1α protein can be independent of the regulation of mRNA expression. A consistent correlation between HIF-1α protein levels and HIF-1 DNA-binding activity have suggested that this sub-unit is the regulatory factor in HIF-1 activity [290]. This has been confirmed using mutation analysis where HIF-1α or HIF-1β sequences have been fused to the DNA binding domain of heterologous transcription factors to create chimaeric genes [292]. In these experiments, sequences from the α sub-unit were found to convey hypoxia-inducibility in transfected cells but no such response was observed for the HIF-1β sequences [292].

It is currently unclear how HIF-1α protein which is synthesised in the cytoplasm, is rapidly translocated to the nucleus in hypoxia [152]. As mentioned, HIF-

1α is not detected in the cytoplasm under normoxia suggesting that it may be rapidly synthesised and degraded in this compartment [152,293]. One suggestion is that hypoxia provides a stabilizing stimulus for HIF-1α since it has been shown that HIF-1α amino acids 549-572, impart protein stability. Once stable, HIF-1α may associate with a cytosolic binding protein which transports it into the nucleus [151]. Although there is currently no evidence for such a mechanism, in the case of AhR (aryl hydrocarbon receptor, a dimerising partner to HIF-1β), the unbound form resides in the cytoplasm as a complex with two molecules of hsp90. Following binding of xenobiotic ligands such as dioxins, the AhR-hsp90 complex passes into the nucleus, where hsp90 (heat shock protein 90) dissociates, allowing AhR to bind to HIF-1β and activate genes involved in xenobiotic metabolism [151]. It has been suggested that hsp90 may also be involved in HIF-1 activation since *in vitro* experiments have shown that the affinity of hsp90 for HIF-1α is even higher than that for AhR [151].

One important feature of HIF-1 is that induction occurs at a physiologically relevant range of O_2 concentration [153]. When HeLa cells were exposed to a range of O_2 concentrations, levels of HIF-1α protein, HIF-1β protein and HIF-1-DNA binding activity increased exponentially as O_2 concentration declined, with a half-maximal response between 1.5-2% and a maximum response at 0.5% O_2 [153]. Measurements of O_2 tension in brain, heart and kidney vary from 3-6% and in the renal cortex specifically, between 3-4%, suggesting that any decrease in O_2 tension *in vivo*, would occur in the steep portion of the HIF-1 response curve, rapidly resulting in a transcriptional response to cellular hypoxia [153].

Hypoxia-responsive sequences have not been previously investigated for the collagen α1(I) gene but examination of murine and human collagen α1(I) promoter sequences (personal communication: Dr.J.Rossert, INSERM, Hopital Tenon, Paris,

France) and published sources [206,281] identified four potential HIF-1 binding sites in both species lying in the region -2000/-1350, (*Fig.5.1A* and *5.1B*). In addition, this region of the human and mouse promoter also contains binding sites for the hypoxia-inducible transcription factors, AP-1 and NF-κB (not shown), suggesting that collagen α1(I) gene transcription may be regulated by hypoxia.

Although HREs have been described for a number of genes [122], the mechanism(s) by which signal of low O_2 is transduced to changes in gene expression are not understood. Two main theories have been proposed [122,294]; the first suggests that the sensor is a haem-protein similar to haemoglobin, in which O_2 is bound to a central atom within a porphyrin ring [295]. The binding of O_2 changes the conformation of the protein from the deoxy-to the oxy- configuration; a decrease in O_2 availability is thought to reverse this conformational change, activating a signal transduction system such as a protein phosphorylation cascade and leading to transcriptional activation [294].

Evidence for such a sensor is provided by the observation that a number of genes can be induced not only by hypoxia but also by certain transition metals (cobalt, nickel and manganese) and by iron chelators such as desferrioxamine [123,296]. Metals such as cobalt are thought to replace the central iron atom within the haem ring; as cobalt has a much lower affinity for O_2 than iron, the protein remains in a 'deoxy' conformation and the haem protein cannot bind O_2 [122,294]. Desferrioxamine is thought to act by chelating iron in the extracellular milieu, making it unavailable for the synthesis of haem proteins which results in inability of the cell to utilize O_2 and thus activates transcription of hypoxia-regulated genes [294]. While an O_2 sensor has now been identified in the regulation of nitrogen fixation genes in *Rhizobium* bacteria, to date no such homologous factor has been identified in mammalian cells [297]. Sequence analysis of the O_2-sensing domain in *Rhizobium* has however revealed sequence

homology with human cytochrome P_{450}, a member of the cytochrome *b*-type haem-protein family and pharmacological studies have shown that modulation of P_{450} can influence hypoxia-induced EPO production in human hepatoma cells [298].

An alternative theory for O_2 sensing suggests that hypoxia can be detected by its influence on intracellular redox status and/or the generation of ROS [122]. Hydrogen peroxide, which is itself generated in an O_2 -dependent manner by cells, can abolish the activation of HIF-1 and induction of EPO and has been implicated as a potential O_2 sensor [299]. In this system, O_2 binds reversibly to the iron atom of a haem protein, which functions as an oxidase, reducing O_2 to superoxide (O_2^-) and peroxide [15]. Free iron generated by this reaction catalyzes the formation of ROS which inactivate HIF-1 by oxidation of sulfhydryl groups. Hypoxia lowers intracellular ROS, reducing sulfhydryl groups and activating HIF-1 as a result [15].

Evidence for a role for hydrogen peroxide as a sensing molecule has been provided by the effect of compounds which perturb hydrogen peroxide production; however, it has not yet been demonstrated that these compounds act within the concentration range of hydrogen peroxide observed in the normoxia to hypoxia transition, thus the evidence for hydrogen peroxide as a sensor is still incomplete [122]. One other molecule which appeared to be an attractive candidate sensor is cytochrome *b558*, a component of the NADPH oxidase complex [300]. This suggestion arose due to the sensitivity of the O_2 sensing system to iodonium compounds which are powerful inhibitors of the NADPH oxidase [301]. However, the finding that HIF-1 is activated by hypoxia in B-cell lines derived from individuals with chronic granulomatous disease, who lack cytochrome b558, excludes the possibility that cytochrome b558 is the sensor [302]. Although all the theories proposed have some merit, no one theory can accommodate all of the available data and the sensor(s) remains to be characterised [303]. In addition, the

signal transduction systems involved in hypoxia-induced gene expression have not been fully elucidated but there is increasing evidence to implicate redox chemistry, changes in intracellular calcium concentration, activation of protein kinase-C, protein kinase-A, tyrosine kinase and protein phosphorylation [15,290,294,303].

Against this background, it was of interest to examine whether the hypoxia-induced increase in collagen α1(I) mRNA observed in *Chapter 2* was a result of direct action on the gene. Detailed examination of the collagen α1(I) promoter identified a cluster of HIF-1 binding sites (*Figs.5.1 A and B*), suggesting that collagen I gene transcription may be responsive to hypoxia. Collagen α1(I) promoter activity was investigated by transient-transfection of collagen α1(I) promoter constructs into 2 cell types and the role of HIF-1 investigated.

5.2 Aims

- To determine the effect of hypoxia on the activity of the collagen α1(I) promoter.
- To examine the role of HIF-1 in increased collagen α1(I) promoter activity.

5.3 Methods

Transient transfection with collagen α1 (I) promoter constructs:

5.3.1 Cell culture

Chinese Hamster Ovary Cells (CHO) cells, a generous gift from Dr. Rachel Fisher (The Atherosclerosis Unit, Karolinska Institute, Stockholm, Sweden) were originally purchased from ECACC. HIF-1α defective CHO cells (Ka13) and the parent wild type cells (CHOwt), were a gift from Dr. Peter Ratcliffe (Institute of Molecular Medicine, John

Radcliffe Hospital, Oxford, UK). CHO and Ka13 were grown in Ham's F12 media (Gibco-BRL) containing 10% FCS (Gibco-BRL) and 1% antibiotic/antimycotic (Gibco-BRL). The medium was changed every 3-4 days and cells passaged at confluence using trypsin-EDTA. NRK-49F, a rat kidney fibroblast cell line was purchased from ATCC. Cells were grown in DMEM: Ham's F12 medium, 1:1 (Gibco-BRL) containing 10% FCS (Gibco-BRL) and 1% antibiotic/antimycotic (Sigma). Medium was changed and cells passaged as for CHO cells.

5.3.2 Collagen α1(I) promoter constructs

Mouse collagen α1(I) promoter constructs containing a luciferase reporter gene were a generous gift from Dr. Jerome Rossert and were modified to excise the first intron (originally placed upstream of the collagen promoter sequence) by Dr. Gisela Lindahl (Centre for Cardiopulmonary Research, RFUCMS, London, UK). Two constructs were generated, one containing -220/+115 bp and the other -2310/+115 bp of collagen α1(I) promoter sequence cloned into the pGL3 basic (pGL3b) luciferase vector (Promega) (*Fig 5.3*). The pGL3b vector (gift from Dr. Lindahl) was used as a control.

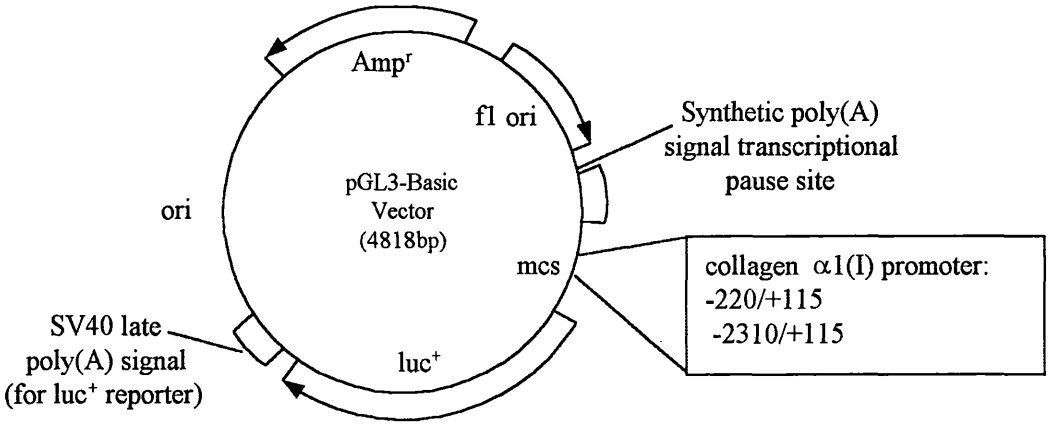

Fig. 5.3 ***Plasmid map of collagen α1(I) promoter constructs.*** Mouse collagen α1(I) promoter DNA containing either -220/+115 or -2310/+115 bp of promoter was cloned into the pGL3-basic vector. Amp^r: gene conferring ampicillin resistance; f1 ori: origin of replication derived from filamentous phage; ori: origin of plasmid replication in E.coli.; mcs: multiple cloning site. Arrows within luc$^+$ and the Amp^r indicate the direction of transcription, the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

5.3.3 Preparation of plasmid DNA

JMI09 bacteria, transformed with plasmid DNA, were received as a frozen stock (gift from Dr. Lindahl). Transformed bacteria were used to inoculate 20ml of LB medium (Sigma) containing ampicillin (50μg/ml; Sigma). Bacteria were incubated for 16hrs at 37°C with vigorous shaking. This starter culture was diluted 1:50 in LB medium containing ampicillin (50μg/ml) and incubated, with shaking, at 37°C until the OD_{600nm} was 0.8. Chloramphenicol (172μg/ml; Sigma) was added to amplify plasmid DNA and cultures were shaken overnight at 37°C. The bacteria were collected by centrifugation at 6,000xg for 15mins at 4°C and the supernatant discarded. Plasmid DNA was prepared using the Qiagen Plasmid Maxi Kit (Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions. The concentration of DNA was measured by absorbance at 260nm and verified by restriction digest analysis: 5μg DNA were digested with 10U of appropriate restriction enzyme and 1X reaction buffer, in a total volume of 15μl (all restriction enzymes and buffers were from Roche Molecular Biochemicals). For pGL3b (4.82kb), BamH1 and HindIII cleaved the DNA to yield 2 fragments of 2.87kb and 1.95kb; for -220/+115 (5.14kb), digestion with XhoI and HindIII yielded fragments of 4.8kb and 335bp and for -2310/+115 (7.2 kb), HindIII digestion generated 2 fragments of 4.8kb and 2.4kb. Digests were incubated overnight at 37°C and DNA fragments separated by agarose gel electrophoresis. Samples were prepared in DNA loading dye (5X stock: 50% glycerol; 1X TBE (20X TBE: Tris base, 1M; Boric acid, 1M; EDTA, 20mM), 1% xylene cyanol, 1% bromophenol blue (all reagents from Sigma) and ethidium bromide (10μg; Sigma). DNA standards (1μg/μl, 1kb ladder; Gibco-BRL) were prepared in DNA loading dye and ethidium bromide (as for samples) and denatured at 65° for 10mins. Samples were electrophoresed on a 1% agarose gel with 1X TAE, pH7.2 (10X TAE: 0.4M Tris-base; 0.2M sodium acetate (Sigma), 10mM

EDTA-$Na_2.2H_20$) as the running buffer. DNA gels were loaded and electrophoresed in 1X TAE for approximately 1hr at 100V. Gels were photographed under UV illumination to visualise the bands.

5.3.4 Transfection

CHO, Ka13 and NRK cells were seeded at a density of $5x10^4$/well in 6-well plates (Greiner) in growth medium and incubated for 16-24hrs. Cells were transfected using Lipofectin® Reagent (1:1 liposome formulation of the cationic lipid N-1[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE), 1mg/ml; Gibco-BRL) according to the manufacturer's instructions). The cell density, Lipofectin® concentration, DNA concentrations and transfection time used were established by optimisation experiments in which the variables were titrated. For transfection, Lipofectin® (4µl/100µl per well for CHO and 6µl/100µl /well for NRK) was prepared in Opti-mem® I Reduced serum medium (Gibco-BRL) and incubated at room temperature for 45mins. DNA was diluted to 2µg/100µl/well in Opti-mem®. Lipofectin® and DNA solutions were then combined, mixed gently and incubated at room temperature for 15mins. Opti-mem® medium (800µl) was added to the Lipofectin®-DNA mixture to give a final volume of 1ml/well. Cells were washed twice with OPTI-MEM® medium and overlaid with the Lipofectin®-DNA mixture. Cultures were incubated for 6hrs at 37°C after which time the transfection medium was replaced with medium containing antibiotic/antimycotic (serum-free for CHO cells, 0.5% FCS for NRK). Cells were incubated for 1hr at 37°C before exposure to hypoxia for 48hrs (parallel transfected cultures were maintained in normoxia) as described in *Chapter 2*: *2.3.4.*

5.3.5 Luciferase Assay

At the end of the hypoxia period, plates were removed from the chamber and immediately placed on ice. Cells were washed twice with ice-cold PBS and scraped into Passive Lysis Buffer (PLB, 400ul/well; Promega). Cells were incubated in PLB for 30mins at room temperature on an orbital shaker. Cell lysates were decanted into microfuge tubes and centrifuged at 15,000xg for 30secs at 4°C to remove cellular debris and the supernatants collected. Luciferase enzyme activity was measured using the Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Briefly, 100µl of Luciferase Substrate Reagent (Luciferase Assay Kit, Promega) was added to 20µl of cell lysate and luminescence measured using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Cell protein/well was measured using a modified Bradford Assay as described earlier (*Chapter 2:2.3.4*) and data expressed as arbitrary luciferase units/mg protein. Data shown are representative experiments of 3 repeats with n=3 wells per DNA construct.

5.3.6. Hirt's Assay

To measure efficiency of DNA transfection, the Hirt's Assay was performed on cultures maintained in normoxic conditions. Cells were transfected as described above and lysed with Cell Lysis Buffer (Roche Molecular Biochemicals UK) for 30mins at room temperature on an orbital shaker. Lysates were centrifuged at 15,000xg for 15mins at room temperature and the nuclear pellet lysed in 0.5ml Hirt's solution (0.6% SDS, 10mM Tris pH 7.5, 10mM EDTA pH 8; all reagents from Sigma) for 10mins at room temperature. After lysis, NaCl (0.5M, final concentration) and proteinase K (0.3mg/ml, final concentration; both reagents from Sigma) were added and samples incubated for 3hrs at 37°C. Samples were centrifuged to pellet cell debris and supernatants mixed with an equal volume of 1:1 phenol-chloroform-isoamyl alcohol (25:24:1 ratio, saturated with 10mM Tris, pH 8, 1mM EDTA;

Sigma) and centrifuged at 15,000xg for 5mins at room temperature. The upper, aqueous phase was collected and 50µl mixed with HCl (0.25M final concentration; Sigma) for 5mins at room temperature. An equal volume of NaOH/NaCl was added (final concentrations 0.6M and 1.4M respectively; both reagents from Sigma) and samples were incubated for a further 15mins at room temperature. Finally 1.4M Tris pH7/1.5M NaCl, was added (final concentrations 1M and 1.1M ; Sigma) and the samples thoroughly mixed by vortexing.

The slot blot apparatus (Minifold II SCR 072/0, Schleicher and Schuell, Dassel, Germany) was assembled with 2 pieces of filter paper (Whatman International Ltd., Maidstone, Kent, UK) pre-wetted with 20X SSC and one sheet of nitrocellulose membrane (0.2µm pore size; Schleicher and Schuell) pre-wetted with water followed by 20X SSC. Samples were applied to slots, allowed to stand for 30mins and the membrane was then vacuum drained. Wells were rinsed with 20X SSC and the blot allowed to air dry. Membranes were baked for 2hrs at 80°C under vacuum prior to Southern analysis using a luciferase probe.

Luciferase probe: A luciferase DNA probe was prepared by cutting vector pGL3b (6µg) with the restriction enzymes NcoI (10U/µl; Roche Molecular Biochemicals) and Xba1 (10U/µl; Roche Molecular Biochemicals) as described for collagen α1(I) plasmid DNA (*5.3.3*). The digest (10µl) and DNA standards (1µg/µl, 1kb ladder; Gibco-BRL) were electrophoresed on a 1% agarose gel (low melting point; Sigma) with 1X TAE as the running buffer. Electrophoresis yielded 2 fragments, 3.17kb vector and 1.66kb luciferase gene. The luciferase fragment was excised from the gel and the concentration of DNA calculated from the total volume of gel obtained. The probe was labelled with α^{32}P-dCTP (3000Ci/mmol; Amersham) by random priming as previously described in *Chapter 2: 2.3.9*.

5.3.7 Southern blot analysis

Membranes were incubated in pre-hybridisation solution (prepared as for Northern Blot analysis, *Chapter 2:2.3.9*) for 2-18hrs at 65°C in a Techne Hybridisation Oven (Techne Ltd.). Blots were hybridised overnight at 65°C in hybridisation solution (see *Chapter 2:2.3.9*) containing 100μg/ml sheared, denatured salmon sperm DNA (Sigma) and 10^7 dpm of α^{32}P-dCTP-labelled cDNA probes. Blots were washed 4 times, 20mins each, in 1XSSC, 0.5% SDS at 50°C with constant agitation and exposed to autoradiography film (Kodak XAR) at -80°C with intensifying screens for 1-6 days. Autoradiograms were scanned and quantified by densitometry (as described in *Chapter 2:2.3.9*) and data expressed as arbitrary densitometry units (*Fig. 5.4*).

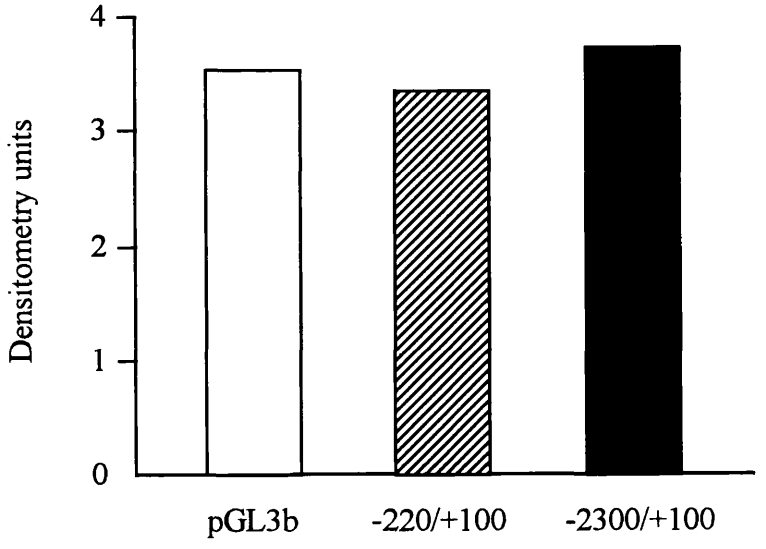

Fig. 5.4. ***Hirt's Assay.*** Cells were transfected with the -220/+115, -2310/+115 collagen α1(I) promoter constructs and the empty vector pGL3b. Cells were lysed and samples applied to nitrocellulose membrane using a slot blot apparatus and the membrane hybridised with a ^{32}P-dCTP labelled luciferase probe. Autoradiograms were scanned and quantitated as described in *Chapter 2*: 2.3.9 and data expressed as arbitrary densitometry units. The data presented are a representative experiment of 3 repeats

The Hirt's assay established that the pGL3b, -220/+115 and -2310/+115 constructs were transfected into cells with equal efficiency.

5.4 Results

5.4.1 Effect of hypoxia on HIF-1 mRNA expression

To examine whether human PTE express HIF-1 mRNA and if levels are altered by hypoxia, Northern blot analysis was performed as described in *Chapter 2* (cDNA for human HIF-1α was a gift from Dr. Graeme Bell (University of Chicago, IL, USA). Since HIF-1α is the unique sub-unit within the HIF-1 complex (HIF-β also heterodimerises with other proteins) and studies in other cell types have suggested that HIF-1α is the regulatory sub-unit of the HIF-1 heterodimer, a cDNA probe for HIF-1α was used [290-292]. In Hep3B cells exposed to hypoxia (1% O_2), HIF-1α mRNA levels are dependent on the duration of O_2 depletion [152]. To evaluate the time-course of HIF-1α mRNA expression, Northern blot analysis was performed on total RNA extracted from PTE exposed to hypoxia for 1, 2, 4, 8 and 24hrs and compared to parallel cultures maintained in normoxic conditions (*Fig. 5.5).*

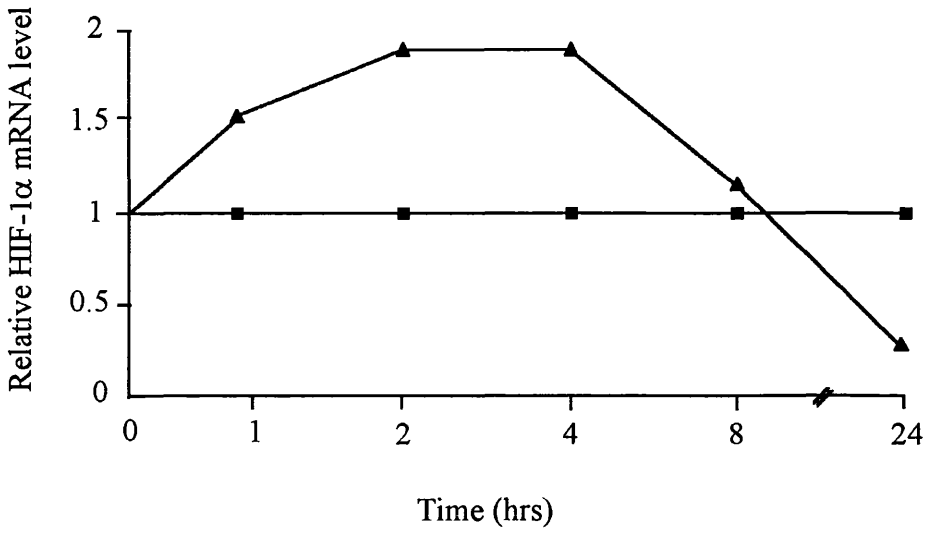

Fig. 5.5 ***Northern blot analysis of HIF-1α mRNA levels in PTE exposed to normoxia (squares) or hypoxia (triangles) conditions for 1,2,4,8 and 24hrs.*** Total RNA was extracted from PTE exposed to 21%O_2 (Normoxia) or 1% O_2 (Hypoxia) for 1,2,4,8 and 24hrs. Autoradiograms were scanned and quantitated as described in *Chapter 2: 2.3.9.* Relative mRNA levels were calculated as fold over control value (assigned value of 1). The data presented are a representative experiment of 2 repeats.

The HIF-1α probe hybridised to a single transcript of ~3.2kb. PTE constitutively express HIF-1α mRNA which was increased after 1hr of hypoxia and reached a plateau between 2-4hrs, after which mRNA levels began to decline.

5.4.2 Transient transfection of collagen α1(I) promoter constructs

To examine whether hypoxia has any effect on collagen α1(I) promoter activity, transient transfection experiments were performed. Two collagen α1(I) promoter constructs containing the luciferase reporter gene were used, one spanning the region -2310/+115, which contains 4 putative HIF-1 binding sites and a smaller construct -220/+115 in which there are no HIF-1 binding sites (*Fig 5.6*).

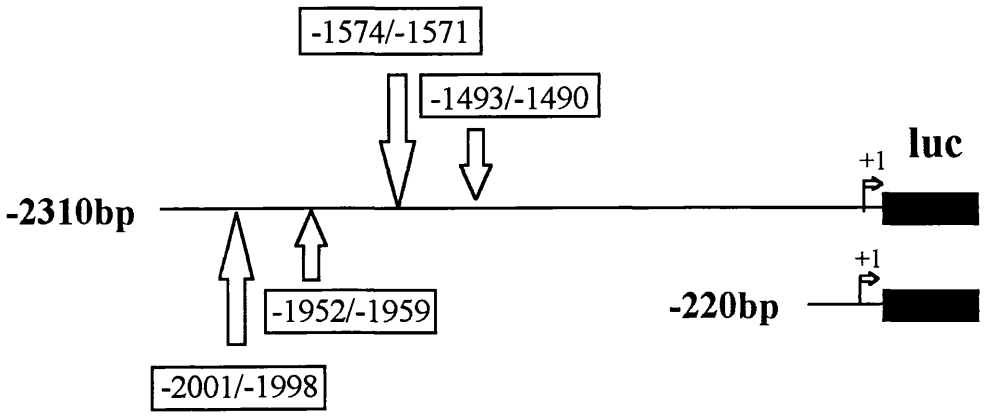

Fig. 5.6 ***Schematic diagram of potential HIF-1 binding sites in the murine collagen α1(I) promoter constructs.*** Arrows indicate HIF-1 core (-CGTG-) binding sites, in the -2310/+115 construct, no -CGTG- sites are present in the -220/+115 construct. For detail of other transcription factors present in the murine COL1A1 promoter, see *Fig. 5.1.B.*

Initially, attempts were made to transfect human primary PTE cells; however only very low efficiency was achieved in these cells, most likely to the low proliferation rate of these cells. It was therefore decided to use CHO cells, as a representative epithelial cell line known to exhibit hypoxia-responsiveness [304,305] which has been extensively used in the study of hypoxia-regulated genes [304,305]. In addition, Wood et al have isolated HIF-1α defective CHO cells (Ka13) which provide a valuable tool for distinguishing HIF-1- mediated and non-HIF-1-mediated hypoxia responses [306].

Both of the collagen α1(I) promoter constructs were transcriptionally active in normoxic cells; with higher basal activity of the -220/+115 construct (*Fig. 5.7 open bars*). Hypoxia induced collagen α1(I) promoter activity in both the -220/+115 and -2310/+115 constructs (*Fig.5.7*). In cells containing the 220/+115 construct (which lacks the putative HIF- binding sites) hypoxia induced a 1.7 fold increase in luciferase activity whilst the -2310/+115 construct was induced by 2.5 fold. On exposure to hypoxia, there was a small, statistically insignificant, induction of empty pGL3b vector.

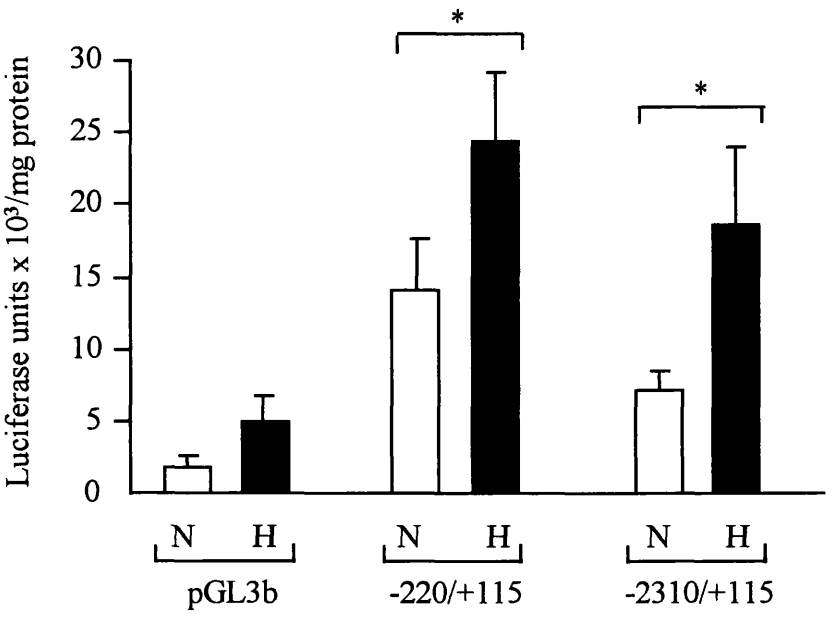

Fig.5.7 ***Luciferase activity in CHO cells transfected with collagen α1(I) promoter constructs and exposed to normoxia (N) or hypoxia (H) conditions for 48hrs.*** Semi-confluent cultures were transfected with PGL3b (control vector), -220/+100 and -2310/+100 collagen promoter constructs for 6hrs and then allowed to recover in serum-free media for 1hr before exposure to normoxia (21% O_2) or hypoxia (1% O_2) for 48hrs. The data shown are a representative experiment of 2 repeats with n=3 per DNA construct. *P<0.05 by unpaired Student's t-test.

To examine whether the collagen α1(I) gene promoter is hypoxia-responsive in other renal cell types, rat renal fibroblasts (NRK-49F) were transfected with the same constructs (*Fig.5.8*). Previous experiments from our group have shown that collagen I mRNA levels are increased in renal fibroblasts exposed to hypoxia of similar duration to that used in experiments on PTE [229].

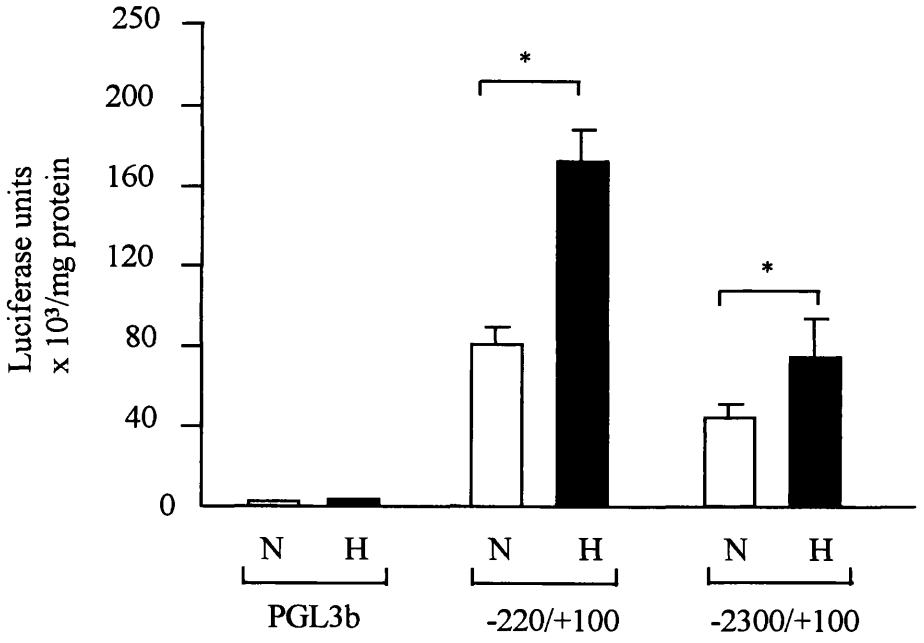

Fig.5.8 ***Luciferase activity of NRK-49F cells transfected with collagen I promoter constructs and exposed to normoxia (N) or hypoxia (H) conditions for 48hrs.*** Semi-confluent cultures were transfected with pGL3b (control vector), -220/+115 and -2310/+115 collagen promoter constructs for 6hrs, allowed to recover in serum-free medium for 1hr before exposure to normoxia (21% O_2) or hypoxia (1% O_2) for 48hrs. Cells were harvested for measurement of luciferase activity and values corrected for cellular protein. The data shown are a representative experiment of 2 repeats with n=3 per DNA construct. *P<0.05 by unpaired Student's t-test.

As in CHO cells, basal promoter activity in normoxia was higher with the -220/+115 construct compared to the -2310/+115. In NRK cells, both promoter constructs were significantly induced by hypoxia, however in contrast to CHO cells, the increase was greater in the -220/+115 construct (2.1 fold increase vs. 1.6 fold induction in the -2310/+115). Also in contrast to the hypoxic-induction of control vector in CHO cells, there was no change in luciferase activity in NRK cells transfected with the empty pGL3b vector.

5.4.3 Transfection of HIF-1α defective CHO cells with -2310/+100 collagen α1(I) construct.

To confirm that HIF-1α is not involved in hypoxia-induced collagen α1(I) gene transcription, Kal13 cells, a mutant CHO cell line which is defective in the HIF-1α sub-unit, was transfected with the -2310/+115 bp construct (*Fig. 5.9*).

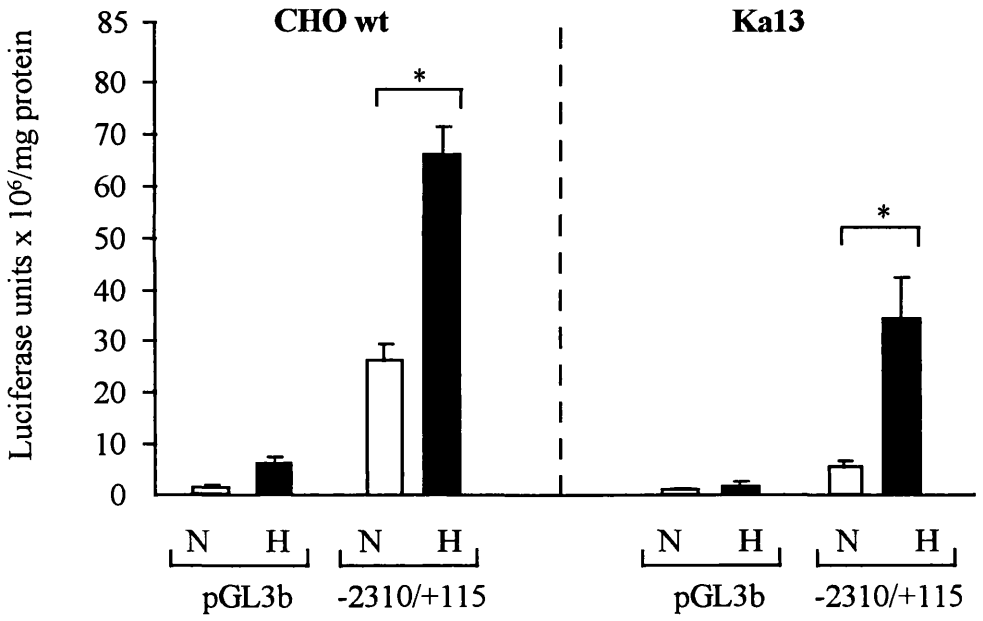

Fig. 5.9 ***Luciferase activity of CHOwt (wild-type) and Kal13 (HIF-1α defective) cells transfected with the -2310/+115 collagen α1(I) promoter construct and exposed to normoxia (N) or hypoxia (H) conditions for 48hrs.*** Semi-confluent cultures were transfected with pGL3b (control vector) and the -2310/+115 collagen α1(I) promoter constructs for 6hrs, allowed to recover in serum-free medium for 1hr before exposure to normoxia (21% O_2) or hypoxia (1% O_2) for 48hrs. Cells were harvested for measurement of luciferase activity and values corrected for cellular protein. The data shown are a representative experiment of 2 repeats with n=3 per DNA construct. *P<0.05 by unpaired Student's t-test.

The -2310/+115 construct was transcriptionally active in both CHO and Ka13 cells (*Fig. 5.9*). As demonstrated previously in Fig. 5.7, hypoxia induced a 2.5 fold increase in luciferase activity in wild-type CHO cells transfected with the -2300/+115 collagen α1(I) promoter construct. Luciferase activity was also induced in Ka13 cells exposed to hypoxia (6 fold). Since Ka13 cells are unable to produce functional HIF-1, but are still

able to induce collagen α1(I) promoter activity, this confirms that hypoxia-induced collagen α1(I) transcription is HIF-1 independent.

5.5 Discussion

The data obtained in studies described in *Chapter 4* demonstrated that the induction of collagen I mRNA by hypoxia is not mediated by a secreted, soluble factor. This raised the possibility that hypoxia may be acting directly at the gene level via one or more hypoxia response elements, as has been described for a number of other genes [120-122]. The collagen α1(I) promoter sequence (-2310/+115) was therefore examined for the presence of potential HIF-1 binding sites and other potential HREs. Both the murine and human collagen α1(I) promoter sequences contain 4 putative HIF-1 binding sites in close proximity (2 of which are conserved between species), the location of which are shown in *Table 5.1*.

Although it is common to find 2 HIF-1 sites situated close together in hypoxia-responsive genes (eg. transferrin, lactate dehydrogenase A, enolase A [133]), a cluster of 4 HIF-1 sites, in close proximity, has not previously been reported in any gene promoter. As can be seen in *Table 5.1*, 2 sites with similar flanking sequences (underlined and double-underlined) are present in both the human and mouse collagen α1(I) promoter and may be of particular importance since they are clearly conserved between species.

Human collagen α1(I) promoter - 2310/+1 region:			
1)	5'-TGGG	CGTG GGAA-3'	-2203/-2200
2)	5'-CCGA	CGTG GCTC-3'	-1694/-1691
3)	5'-AGGG	CGTG GAGA-3'	-1622/-1619
4)	5'-AGGA	CGTG GAGT-3'	-1379/-1376
Murine collagen α1(I) promoter - 2310/+1 region:			
1)	5'-CGCG	CGTG TGTG-3'	-2001/-1998
2)	5'-GCTT	CGTG NCAT-3'	-1952/-1949
3)	5'-GCGA	CGTG GCTC-3'	-1574/-1571
4)	5'-AGGG	CGTG AAGG-3'	-1493/-1490

Table 5.1. ***Potential HIF-1 binding sites and their location on the human and murine collagen α1(I) promoter.*** Sequences underlined and double underlined are identical in the two species. Table compiled from sequences obtained from Dr.J.Rossert and from published sources [206,281].

Comparison of these HIF-1 sequences with HIF-1 binding sites of other hypoxia-responsive genes showed sequence homology only in the core region -CGTG-; the flanking sequences do not match those of any other HIF-1 sites. It is interesting to note however that an A base 5' to the core sequence is common in HIF-1 binding sites and that two of the human sites and one of the mouse sites in the collagen α1(I) promoter have this -(A)CGTG- sequence.

The presence of potential HIF-1 binding sites suggested that hypoxia may act directly via these elements to upregulate collagen α1(I) gene transcription; although the presence of these sites do not guarantee functional involvement since inactive HIF-1 sites

have been identified in several genes [151] and other transcription factors can mediate hypoxia-responses [154,155,158,159]. In addition, it has been demonstrated that the transcription factors ATF and CREB can constitutively bind to the HIF-1 core sequence of the EPO gene [157]. Nevertheless, it is of interest that the collagen α1(I) promoter contains multiple HIF-1 binding sites which may potentially act in concert. Although the hypoxic-induction of genes such as transferrin requires the interaction of 2 HIF-1 sites [133], a hypoxia response requiring multiple HIF-1 sites has not been described.

Since HIF-1α is the unique HIF-1 sub-unit and the inducible component which mediates gene regulation, PTE HIF-1α mRNA levels were examined after exposure to hypoxia for 1-24hrs as a marker of the hypoxic response. As observed for Hep3B cells, there was a rapid, transient increase in HIF-1α mRNA which peaked between 2-4hrs then declined rapidly to basal levels. Although increased HIF-1α and β mRNA levels in response to hypoxia have been reported in some cell types [152]; induction of HIF more frequently appears to be post-transcriptional or due to increased DNA-binding activity [289]. It has been suggested that on exposure to hypoxia, HIF-1 mRNA induction is small or absent in cells where HIF-1 is basally expressed [290]; in PTE however, HIF-1α mRNA was transiently induced despite constitutive expression.

The hypoxic induction of HIF-1α mRNA by PTE demonstrates that HIF-1 gene expression is induced by hypoxia in this cell type but does not prove that HIF-1 is involved in the induction of the collagen α1(I) gene. One hypoxia-inducible gene in PTE which may be regulated by HIF-1 is GAPDH [127](*Chapter 2, Fig 2.7*); it is therefore possible that increased HIF-1α mRNA levels reflect the involvement of HIF-1 in hypoxic-induction of GAPDH and other genes regulated by O_2 in PTE and are unrelated to regulation of collagen α1(I).

To investigate the hypoxia-inducibility of the collagen α1(I) promoter, two 5' constructs were tested containing either -2310/+115 or 220/+115bp. The larger -2310/+115bp construct contains all 4 HIF-1 binding sites while the smaller -220/+115bp construct lacks any consensus HIF-1 binding sites. The initial aim was to use the human PTE in transient transfection experiments, however the low proliferation rate of these cells resulted in very low transfection rates. Since the goal of these studies was to determine whether the collagen α1(I) promoter is hypoxia-responsive, 2 cell lines were selected; the CHO cell line as a representative epithelial cell line in which hypoxia-responsiveness has been well characterised and a renal fibroblast cell line NRK-49F. The NRK fibroblasts are currently being used in parallel studies in our laboratory to analyse TIMP-1 promoter regulation in response to hypoxia [307].

In both CHO and NRK-49F cells, both collagen α1(I) constructs were transcriptionally active under normoxic conditions. Activity was greatest in the -220/+115 construct; since both collagen promoter constructs were transfected with equal efficiency (see *Fig. 5.4*) this suggests that the -2310/+115 construct may contain negative regulatory elements which are absent in the 220/+115 sequence.

In CHO cells, hypoxia induced both the -220/+115 and -2310/+115 constructs. This suggests that, in this cell type, HIF-1 may not be the only transcription factor involved in hypoxia-regulation of the collagen α1(I) promoter. One possibility is that hypoxia-responsive sequences (non-HIF binding) in the -220/+115 construct interact with the HIF-1 sites present in the -2310/+115 to enhance transcriptional activation. In addition to the HIF-1 binding sites in the larger construct, examination of the murine promoter (*Fig.5.1A*) identified an AP-1 site at -675; since AP-1 is known to be hypoxia-inducible in HeLa cells it may also be of importance in this response [158]. Although the -220/+115 construct does not contain any HIF-1 binding sites, it contains sequences

which bind many other transcription factors (some of which are shown in *Fig.5.1*) that may be hypoxia responsive (discussed further in *Chapter 7*).

Hypoxia also induced both -220/+115 and -2310/+115 promoter activity in NRK cells but in this cell type the response was greater in the -220/+115 construct. Since there are no HIF-1 binding sites in the -220/+115 promoter construct, other, as yet unidentified hypoxia-inducible transcription factor binding-sites must be responsible for this induction. The lower activity of the -2310/+115 construct suggests that it may contain negative regulatory elements which repress the hypoxia-responsive sites present in the smaller construct. As in CHO cells, induction of the -220/+115 construct by hypoxia suggests that transcription of the collagen α1(I) promoter is not primarily HIF-1 mediated.

These data suggest that collagen α1(I) transcription is not dependent on HIF-1. To confirm this, Ka13 cells were transfected with the -2310/+115 construct. Ka13 are a mutant CHO cell which is functionally defective in the α-subunit of HIF-1 [306], possibly resulting from a frame shifting mutation of the HIF-1α gene that prevents correct translation and leads to the production of an unstable mRNA. Studies from our group have demonstrated that while GAPDH expression is induced by hypoxia in wild-type CHO cells, levels are not altered by hypoxia in Ka13, confirming that the HIF-1α mutation results in loss of function (unpublished observations). In normoxia, there was basal activity of the -2310/+115 construct in both CHOwt and Ka13 cells. Hypoxia increased activity of the -2310/+115 construct in CHOwt cells (as previously observed) but this induction was not reduced in Ka13, as would be expected if collagen α1(I) transcription was HIF-1 mediated. Instead, the fold increase in luciferase activity in Ka13 was even greater than in CHO (2.5 vs 6 fold respectively), confirming that the effect of hypoxia on collagen α1(I) gene transcription is independent of HIF-1. The

mechanism of hypoxia-induced collagen α1(I) gene transcription therefore remains unknown and future studies should be focussed on identifying the *cis*-elements and *trans*-acting factor(s) which mediate the hypoxic response.

5.6 Summary and conclusions

- Examination of both the murine and human collagen α1(I) promoters identified the presence of 4 putative HIF-1 core binding sequences within the region -2000/-1350, suggesting possible HIF-1 involvement in hypoxia-induced collagen I mRNA expression in PTE.
- The HIF-1α mRNA time course demonstrated that in PTE cells exposed to hypoxia there is a rapid induction of expression, suggesting that HIF-1 may be involved in mediating hypoxia-induced gene transcription in PTE.
- Transient-transfection of collagen α1(I) promoter constructs in both CHO cells and NRK-49F demonstrated that the collagen α1(I) gene promoter is responsive to hypoxia.
- Increased activity of both the -220/+115 (without HIF-1 sites) and the -2310/+115 constructs (with HIF-1 sites) in CHO and NRK-49F cells exposed to hypoxia suggested that increased transcription is independent of HIF-1. Confirmation of this was obtained from the finding that hypoxia increased activity of the -2310/+115 construct in Ka13, a CHO mutant cell line defective in HIF-1α. Thus the hypoxia-induced transcription factors which mediate collagen α1(I) gene induction remain to be identified.

CHAPTER 6

Conclusions

The mechanisms underlying the initiation and progression of TIF remain obscure [5]. Since fibrotic renal diseases of diverse etiology are commonly associated with vascular compromise, it was hypothesised that tissue hypoxia, either alone or in conjunction with other factors, may be a pro-fibrogenic stimulus [11]. Although all cellular components of the tubulo-interstitium (PTE, capillary endothelia and interstitial fibroblasts) are likely to be affected by decreased oxygen availability, the metabolic functions of PTE confer high oxygen demands, rendering these cells particularly vulnerable to fluctuations in oxygen levels *in vivo* and leading to the suggestion that tubular cells may be the primary target of a hypoxic insult [13,14]. Changes within proximal tubules occur early in the pathogenesis of TIF with the striking appearance of a dense, thickened TBM surrounding hypertrophic and subsequently, atrophic, non-functioning tubules [1]. This expanded TBM with altered matrix composition presumably has profound influence on PTE function and on paracrine interactions with other cell types; however the pathological stimulus(i) for these changes in the TBM is currently unclear. This project investigated the possibility that hypoxia may be a fibrogenic stimulus for PTE, eliciting changes in ECM metabolism (*Fig. 6.1*).

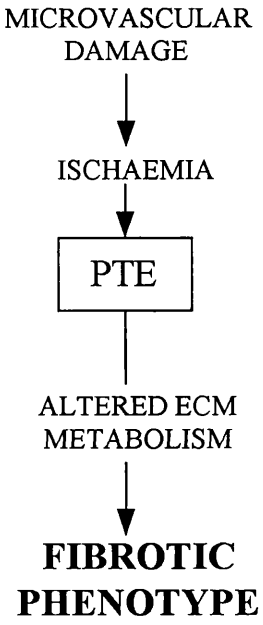

Fig. 6.1 Flow diagram demonstrating the potential effect of ischaemia, arising from microvascular damage, on ECM metabolism in PTE.

The effects of hypoxia on PTE were examined using an *in vitro* model with cells grown in monolayer. Although the limitations of this approach are recognised, this culture system allowed the examination of hypoxia *per se* without the multiple interacting factors present *in vivo*. Preliminary attempts were made to undertake experiments with PTE cultured in 3D collagen gels, since there is some evidence that PTE can form tubular structures when embedded in collagen-I, thus resembling their *in vivo* configuration. It was foreseen that such a model would allow examination of hypoxia-induced changes in PTE cultured alone and also in co-culture with other tubulo-interstitial cell-types, thus giving some insight into the effect of hypoxia on paracrine interactions between renal cells. Despite many attempts to perfect the 3D culture system however, the model proved difficult to manipulate, with retrieval of cells almost impossible. It was therefore decided to use simple monolayer culture, which still allows co-culture with additional cell types grown on tissue cultue inserts, as a representative model of the *in vivo* environment.

The degree of hypoxia chosen for experiments was based on the knowledge that under normal physiological conditions, cortical oxygenation levels are between 3-5% and that levels are likely to vary from this range, down to near anoxia in the event of microvascular damage [153]. PTE were subjected to $1\%O_2$ for 24hrs (*Chapter 2*), conditions which are known to induce expression of EPO and various growth factors in other cell types, *in vitro* [123]. Since this degree of hypoxia had no effect on cell viability, but was sufficient to elicit changes in PTE protein synthesis and cellular respiration (as indicated by elevated GAPDH expression), this level of oxygenation was considered appropriate for the study of hypoxia induced ECM metabolism in PTE.

As discussed in *Chapter 3*, the accumulation of ECM material within the TBM in fibrosis results from increased synthesis and/or decreased degradation of matrix proteins [5]; hypoxia had marked effects on both aspects of matrix metabolism in PTE. Despite an overall suppression in total protein production (perhaps reflecting the decrease in cellular ATP available for protein synthesis), collagen production was stimulated approximately 5-fold by exposure to hypoxia for 24hrs, suggesting a specific effect of hypoxia on collagen proteins. Re-oxygenation, an *in vitro* analogy of reperfusion, further stimulated collagen production and continued to increase levels for up to 72hrs (*Chapter 3*), suggesting a long term effect of hypoxia on collagen protein despite the return to normal oxygen levels.

Since collagen IV is the major collagenous component of the TBM, it was of interest to determine whether hypoxia induced increased collagen production was due to an increase in production of collagen IV. Surprisingly, collagen IV mRNA levels decreased after hypoxia and remained suppressed on re-oxygenation, demonstrating for the first time, the (down) regulation of this collagen sub-type by oxygen depletion. In contrast, mRNA for the interstitial collagen, collagen I, was markedly increased by

hypoxia and remained elevated on return to normoxia, suggesting that increased collagen production in response to hypoxia was due to the induction of interstitial rather than BM collagen. A similar increase in expression of collagen I, accompanied by decreased collagen IV levels, has been reported in *in vivo* models of fibrosis [50]. Since matrix proteins act as regulators of cell growth, differentiation and function, qualitative and quantitative changes in TBM are likely to have profound effects on tubular cell behaviour.

ECM accumulation in TIF is partly attributed to decreased matrix turnover [5], therefore it was of interest to examine the effects of hypoxia on the MMP pathway. Hypoxia decreased both latent and active MMP-2 and MMP-9 enzymes and levels remained on re-oxygenation. While there is some *in vitro* evidence that MMP-9 is regulated by hypoxia in keratinocytes [166], this is the first demonstration of altered MMP-2 activity in any cell type. MMP-2 mRNA levels were not affected by hypoxia in PTE suggesting a translational or post-translational level of regulation. Examination of endogenous MMP inhibitor (TIMP-1 and -2) levels in response to hypoxia, showed increased TIMP-1 secretion but no change in mRNA expression, implying post-transcriptional regulation of the inhibitor in this period. In contrast, TIMP-2 mRNA levels were transiently suppressed during hypoxia demonstrating reciprocal effects of hypoxia on the two inhibitors. Both TIMP-1 and -2 mRNA levels were increased in the re-oxygenation phase.

The data in *Chapter 3* demonstrate that *in vitro,* hypoxia acts as a pro-fibrogenic stimulus for PTE by increasing collagen production while simultaneously decreasing levels of matrix degrading enzymes and increasing inhibitor levels. Similar effects of hypoxia *in vivo* would potentially lead to an accumulation of matrix with clear repercussions on tubular cell function. In addition, thickening of the TBM leads to

physical separation of tubules and capillaries, increasing the oxygen diffusion distance, and thereby subjecting the tubule to further ischaemia. Furthermore, increased metabolic activity of PTE induced by increased filtered protein in the diseased kidney may exacerbate hypoxia and hence lead to further ECM deposition, maintaining a cycle of progressive, TBM thickening (*Fig. 6.2*).

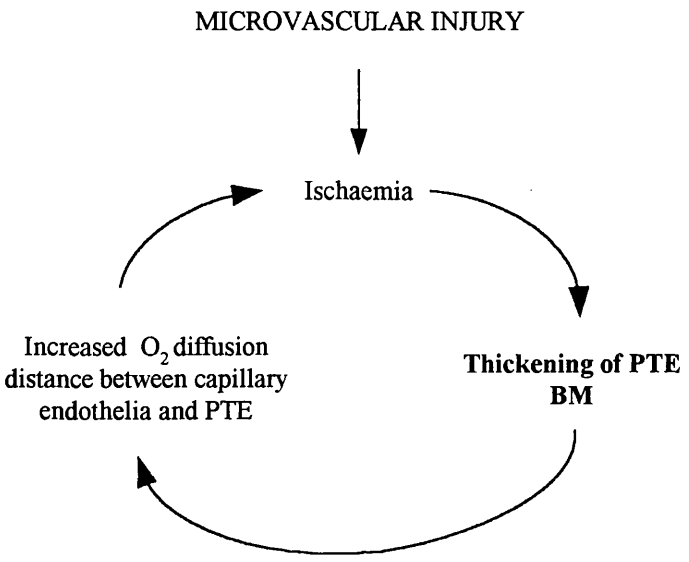

Fig. 6.2 Cycle of progressive thickening of TBM with ischaemia.

These data suggest that even if normal oxygenation is restored ie. by microvascular regeneration, ECM accumulation may progress, although it is not clear whether enzyme, inhibitor and/or ECM protein levels would, with time, return to control or if the changes induced by relatively short periods of hypoxia can lead to long-term alterations and a chronic accumulation of ECM material. While a 24hr period of 1% O_2 is unlikely to have a direct *in vivo* parallel, these studies demonstrate the potential for hypoxia-induced changes in ECM PTE metabolism.

In vivo, the TBM separates PTE from adjacent fibroblasts and the capillary endothelial BM [21]. The close apposition of the different cell types within the tubulo-

interstitium and the observation that fibrosis affects all of these compartments suggest that this pathology may arise through a complex array of cell-cell and cell-matrix interactions. Qualitative and quantitative changes in the TBM, will presumably, not only influence tubular cell function, but are likely to have a marked effect on interactions with adjacent cell types; for example, studies from our laboratory have demonstrated that CM from PTE grown on different substrates can differentially regulate proliferation and MMP expression in interstitial fibroblasts, highlighting the importance of BM composition in modulating cellular interactions [24].

In attempting to understand the mechanism(s) by which hypoxia exerts its effects on PTE ECM metabolism, two possibilities were considered; either the changes were due to an 'indirect' mechanism acting via hypoxia-induced factors or the effects were the result of specific 'direct' effects on matrix genes or genes regulating ECM turnover [120-122], analogous to the direct effects of O_2 depletion on expression of EPO. Initially, the first possibility seemed most likely since PTE produce many pro-fibrogenic growth factors, some of which have been shown to be induced by hypoxia in other cell types [123]. Of such factors, a strong candidate was the premier fibrogenic cytokine TGF-β1; the mediator implicated in hypoxia-induced collagen I production in mesangial cells and dermal fibroblasts [163,164]. Surprisingly, despite an elevation in TGF-β1 protein with hypoxia, neutralising antibody studies demonstrated that it was not the mediator of hypoxia-induced changes in collagen I or MMP-2 activity (*Chapter 4*). In addition, exogenous TGF-β1 failed to mimic the effects of hypoxia on other aspects of the response, suggesting that these changes are also independent of TGF-β1.

The elimination of TGF-β1 as a potential mediator, raised the possibility that some other hypoxia-inducible growth factor was responsible for the changes induced by O_2-depletion. Expression of PDGF and VEGF was induced by 24hrs hypoxia in PTE,

demonstrating for the first time that these growth factors are regulated by hypoxia in this cell-type, *in vitro*. Despite the induction of PDGF and VEGF in hypoxia, neither of these factors had any effect on MMP-2 activity when added exogenously. Previous experiments from our group showed that ET-1 is induced by 48hrs hypoxia in PTE [182] but again, addition of ET-1 to PTE cultures at a range of concentrations, had no effect on MMP-2 activity, suggesting that this also was not the mediator. The role of increased TGF-β1, PDGF, VEGF and ET-1 levels in response to hypoxia therefore remain unknown; it is possible that they exert yet undefined autocrine actions on PTE such as changes in cell adhesion. Induction of hypoxia-induced growth factors by PTE, *in vivo*, would potentially have potent effects on other cell types within the tubulo-interstitum. TGF-β1, is known to have marked effects on fibroblast ECM metabolism; our group has demonstrated increased expression of collagen I mRNA and elevated TIMP-1 levels in response to TGF-β1 [229]. In addition, TGF-β1 induces α-SMA, a marker of activation to a fibrogenic, myofibroblastic phenotype [229]. Secretion of TGF-β1 by PTE in response to ischaemic injury may therefore indirectly contribute to the accumulation of ECM within the interstitium in TIF via action on neighbouring fibroblasts. Similar effects on fibroblast ECM have also been described for PDGF-BB, another pro-fibrogenic factor upregulated by PTE in response to hypoxia [5,123]. Factors secreted by PTE in response to hypoxia may also regulate microvascular endothelial cell function. VEGF has potent effects on endothelial cell permeability, and may act on peritubular capillaries, resulting in further microvascular injury and ischaemia within the tubulo-interstitium [263]. Although not examined in this study, previous data from our group has shown that, secretion of the vasoconstrictor ET-1 is stimulated by exposure of PTE to 48hrs hypoxia, possibly resulting in narrowing of nearby vessels with a decrease in blood flow and further lower tubulo-interstitial oxygenation (*Fig.6.3*).

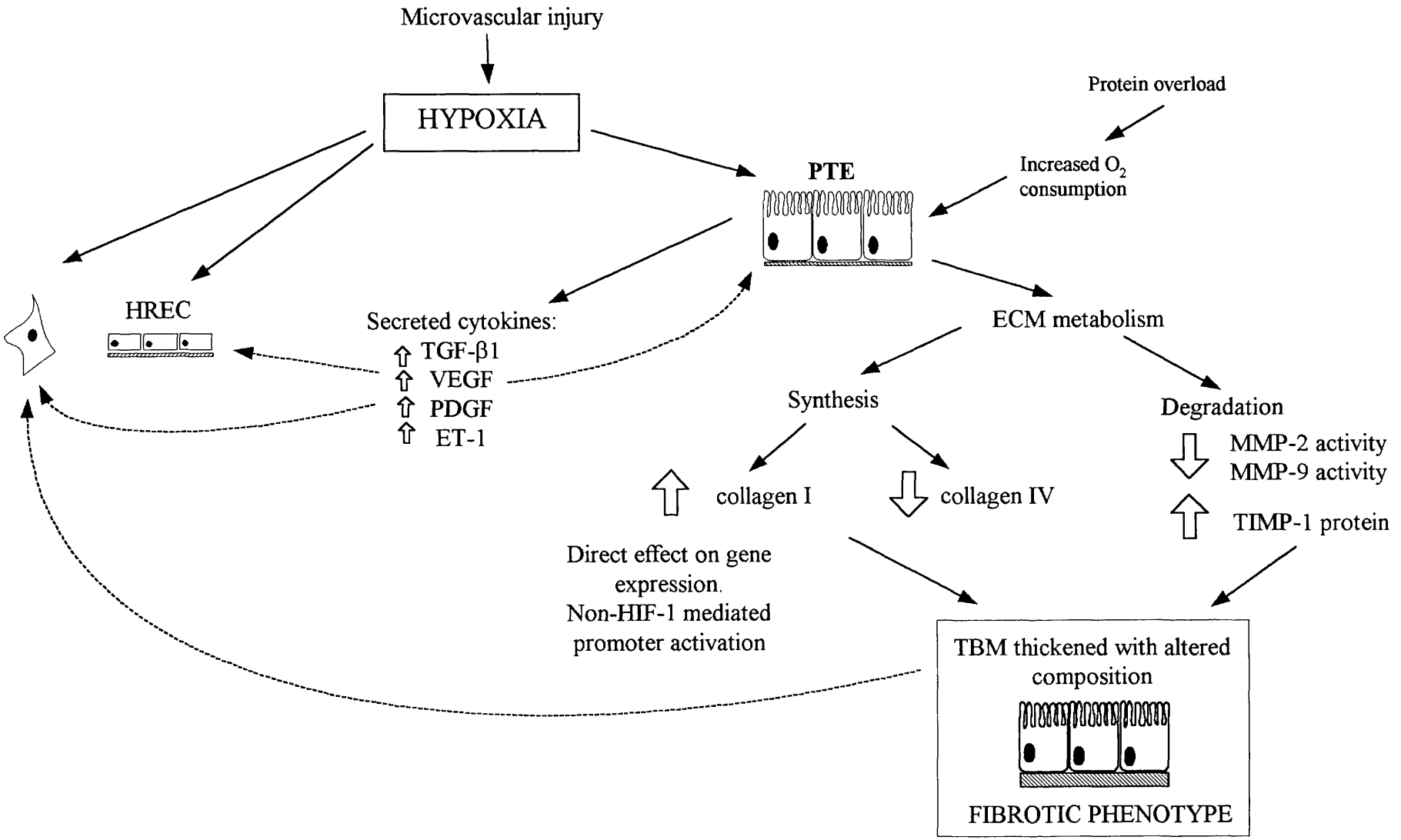

Fig. 6.3 ***Schematic summary of the effects of hypoxia on PTE ECM metabolism and growth factor production in vitro***. Potential autocrine and paracrine interactions in vivo are indicated by a dotted arrow.

The investigation of several candidate growth factors did not identify a mediator(s) of the hypoxia-induced changes in ECM metabolism. Therefore rather than proceed with the time consuming task of examining individual factors, an alternative approach to this question was adopted. Conditioned medium transfer experiments were used to establish whether soluble secreted factors could induce the hypoxic phenotype in PTE. The addition of hypoxic PTE-CM to naive PTE had no effect on collagen I mRNA levels, confirming that the induction in collagen I was not mediated by a stable, soluble, secreted factor. This raised the intriguing possibility that hypoxia has a 'direct' effect on the collagen I gene via one or more hypoxia response elements (HREs). Examination of the collagen α1(I) promoter, identified 4 HIF-1 binding sites between -2000/-1350 bp, suggesting a possible HIF-1 mediated mechanism, as described originally for EPO [295]. HIF-1 mRNA levels were rapidly induced by hypoxia in PTE. Although not conclusive of HIF-1 mediated collagen I gene induction, this data did provide some evidence that hypoxia can elicit HIF-1 mediated responses in PTE subjected to hypoxia.

Transient transfection studies were used to examine whether hypoxia had any effect on the collagen α1(I) promoter. For transfection, two luciferase reporter constructs were used containing: i) -2310/+115bp (with HIF-1 sites) and ii) -220/+115bp (without HIF-1 sites) of the collagen α1(I) promoter. Since attempts to transfect PTE were unsuccessful (probably due to the low proliferation rate of these cells) and the objective of this part of the project was to determine whether the collagen α1(I) promoter is regulated by hypoxia, it was decided to use CHO cells as an alternative epithelial cell type. Hypoxia increased the activity of the -2310/+115bp construct, demonstrating for the first time that hypoxia has a 'direct' effect on the collagen α1(I) promoter. Interestingly, this increase in induction persisted on transfection of the smaller construct, suggesting that the effect is independent of HIF-1. Similar results were

obtained from transfections of NRK-49F cells (a renal fibroblast cell line), suggesting that this effect is not cell-type dependent. The use of a CHO cell line defective in HIF-1α [306] confirmed that the stimulation of collagen α1(I) promoter activity in hypoxia does not require the action of HIF-1; therefore the HRE(s) present in the collagen α1(I) promoter and the transcription factors which bind to these, remain unknown (*Chapter 7*).

The demonstration that hypoxia has direct effects on the collagen α1(I) promoter has important implications for PTE in TIF. If the effects of ischaemia *in vivo* mimic the *in vitro* changes described, the direct action of hypoxia on the collagen I gene would provide a rapid means of inducing this matrix protein within the TBM, independent of a secondary mediator such as a growth factor. Many genes which are hypoxia-responsive are involved in homeostatic processes which must proceed at a rapid rate eg. EPO, the hormone regulating maturation of red blood cells [132,144] and enzymes regulating glycolysis such as GAPDH [127]. A similar rapid induction of collagen I by PTE in response to hypoxia would very quickly alter the composition of the TBM, with repercussions on tubular cell function and interactions with neighbouring cell-types.

Due to their high metabolic demands, it is likely that PTE are a major target for ischaemic injury, however parallel studies from our group and preliminary experiments in this study demonstrate that hypoxia can elicit fibrogenic responses in other cell types found in the interstitium [229,307,308]. Conditionally immortalised, temperature-sensitive, human cortical fibroblasts (CF) exposed to 1% O_2 for 24hrs increased gene expression for collagens I and III, and TIMPs-1 and -3. In this cell type, MMP-2 levels were not affected by low oxygen but both mRNA and protein for interstitial collagenase, MMP-1, were suppressed [229]. The increase in collagen I mRNA levels in interstitial fibroblasts occurs independent of TGF-β1[229] and since collagen I promoter activity is

also induced in NRK fibroblasts, one can speculate that the increase in interstitial collagen I levels observed in TIF may arise from the direct effect of hypoxia on interstitial fibroblasts. An additional effect of hypoxia on CF is stimulation of α-SMA expression, inducing a myofibroblastic phenotype [229]. If CF were to respond to ischaemic injury in a similar fashion *in vivo*, then hypoxia may also provide the stimulus for ECM accumulation within the interstium in TIF.

In human renal microvascular endothelial cells (HREC; a generous gift from Dr.T.Daniels Vanderbilt University Nashville, TN, USA), ECM metabolism is also modulated by hypoxia [308]. As in PTE, both MMP-2 and MMP-9 levels were decreased and collagen IV levels suppressed by hypoxia [308]. One feature of diseases associated with TIF is vascular sclerosis, characterised by thickening of capillary BM [28]. Therefore one can speculate that HREC subjected to O_2 depletion in the kidney would undergo changes resulting in increased ECM deposition, leading to thickening of arteriolar walls. This would presumably increase the O_2 diffusion distance, resulting in further hypoxia to the tubulo-interstitium. Taken together these experiments demonstrate that although hypoxia has slightly different effects on ECM metabolism in the major tubulo-interstitial cell types, the overall effect is pro-fibrogenic.

One of the features of fibrotic diseases is their progressive nature [10]. It is not currently understood why in some pathologies where the initiating injury is well defined, removal of the injurious stimulus does not lead to resolution of the disease [9,10]. This suggests that the initial injury sets in train a cascade of events which cannot be halted. In this respect, fibrosis has been described as a pathological wound healing response [10]. Deposition of ECM material is an essential feature of normal wound repair but usually ceases once healing is complete [309]. For reasons that remain obscure, accumulation of ECM material in fibrosis becomes continuous, escaping control points usually present in

normal wound healing [10]. The concept of ischaemia as an initiating factor in TIF may explain this progression. If hypoxia induces thickening of the TBM, expansion of the interstitial compartment and sclerosis of capillary walls via dysregulation of ECM metabolism in these cells, these events would further increase the O_2 diffusion distance between capillaries and other cell types. Presumably this would exacerbate ischaemic injury, leading to further ECM accumulation, ultimately resulting in a cycle of relentless, progressive fibrosis.

In summary, the data from this study support the hypothesis that hypoxia arising from microvascular damage may provide a crucial link between the initiating glomerular injury and the thickening of TBM (matrix accumulation) observed in progressive fibrosis. Qualitative and quantitative changes in TBM resulting from ischaemic injury, may trigger a cascade of events in the tubulo-interstitium, altering both tubular cell function and paracrine interactions with neighbouring fibroblasts, capillary endothelia and inflammatory cells (*Fig. 6.3*). The net effect of hypoxia on tubulo-interstitial cells is ECM accumulation, which provides a physical barrier for oxygen diffusion, leading to further ischaemia, matrix deposition and, ultimately, renal failure. The concept of hypoxia as a modulator of matrix metabolism is clearly of importance in the kidney but is also likely to have relevance in other organs which undergo fibrosis such as the lung, liver and skin where ischaemic injury precedes the development of fibrosis [297,310,311].

In conclusion, the aim of this study was to investigate the effect of hypoxia on ECM metabolism in PTE within the context of TIF. These data demonstrate that hypoxia alters both ECM synthesis and turnover in PTE and induces the expression of a non-basement membrane collagen, collagen I. Similar changes in matrix composition and turnover in vivo could alter both tubular cell function and paracrine interactions with

other cell types leading to ECM accumulation. The data presented therefore support the hypothesis that hypoxic injury to PTE is a pro-fibrogenic stimulus and may provide a common pathway by which diseases of diverse etiology produce a similar pathology. The demonstration that hypoxia has direct effects on the collagen α1(I) gene may provide an avenue for future therapeutic intervention in fibroses associated with microvascular injury.

CHAPTER 7

Future Studies

Data from this project provides the basis for many future studies both to further examine the profibrogenic effects of hypoxia in PTE and to investigate the molecular mechanisms by which these effects are mediated. Three main lines of investigation are proposed.

- **Identification of the hypoxia response element(s) in the collagen α1(I) promoter**

The data in *Chapter 5* demonstrate that the collagen α1(I) promoter region spanning -220/+115 is stimulated by hypoxia. Since this region does not contain HIF-1 binding sites, the hypoxia-responsive transcription factor(s) responsible for this induction remain unknown. Further studies should focus on identifying these factors and the DNA sequences on the collagen α1(I) promoter to which they bind. One approach in identifying the HRE(s) present on the collagen α1(I) promoter is sequential deletion of the -220/+115 construct (approx 10-20bp intervals) from the 5' and 3' ends, ligation of the fragments into the pGL3b vector and transient-transfection of the different constructs into CHO cells to test hypoxia-inducibility under normoxic and hypoxic. In parallel with this, DNA footprinting could be used to investigate the transcription factors which bind to this region. Once hypoxia-responsive sequences are identified, if these elements bind known transcription factors, gel shift and super shift assays and single base mutations through the region could be used to confirm hypoxia-induced DNA binding proteins. It is possible that novel transcription factors/complexes might be identified.

Examination of the -220/+115 sequence identified the presence of specific motifs which may be involved:

1. AP1 binding site at -102/-96. AP1 is upregulated by ROS generated in hypoxia and re-oxygenation
2. Several Sp1 binding sites. Sp1 and Sp3 transcription factors have been implicated in hypoxia-responsiveness of the β-enolase and pyruvate kinase M genes.

- **Mechanism of suppression in collagen IV mRNA levels with hypoxia**

Few genes are inhibited by hypoxia (*Chapter 5*), therefore it is of particular interest that collagen IV mRNA levels are suppressed when PTE are subjected to hypoxia. In preliminary experiments, a similar finding was observed in human renal endothelial cells suggesting that this may be a phenomenon of cells which produce a BM. Further experiments could be undertaken to examine whether the decrease in steady state mRNA levels is due to decreased message stability or decreased gene transcription in response to hypoxia. A similar approach to that adopted for examination of the collagen α1(I) promoter could be utilised to identify potential repressor elements in the collagen IV gene which inhibit transcription in hypoxia.

- **Examination of post-transcriptional MMP-2 regulation in response to hypoxia**

The data in *Chapter 3* demonstrate that MMP-2 levels in PTE are suppressed by hypoxia with no change at the mRNA level. This suggests that hypoxia interferes with MMP-2 translation or secretion. Protein translation can be inhibited by translation repressor proteins that bind near the 5' end of the mRNA where translation would

otherwise begin, however it is not known if such proteins are regulated by hypoxia. One other possibility is that hypoxia interferes with the exocytosis apparatus responsible for secretion of MMP-2. Alternatively secretion may be inhibited by the low availability of ATP levels during hypoxia.

Additional lines of investigation could include:

- examination of the effects of i) longer periods of less severe hypoxia, in an attempt to mimic sustained chronic low grade hypoxia *in vivo* and ii) repeated hypoxic insults to establish whether a single hypoxic episode renders the cell more susceptible to a second hypoxic 'hit' resulting in sustained injury.
- Examination of the signalling mechanisms involved in the hypoxia-induced increase in collagen I mRNA.
- Investigation of the effects of hypoxia on integrin expression in PTE.
- Co-culture of hypoxic PTE with other tubulo-interstitial cell-types to investigate whether changes in PTE induced by hypoxia alter paracrine interactions leading to fibrogenic changes in adjacent cell-types.

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