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HORMONES - CYTOKINES - SIGNALING

Hypoxia stimulates proximal tubular cell matrix production via a TGF-β_1-independent mechanism

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Hypoxia stimulates proximal tubular cell matrix production via a TGF-β_1-independent mechanism. Tubulointerstitial fibrosis is characterized by tubular basement membrane thickening and accumulation of interstitial extracellular matrix (ECM). Since chronic low-grade hypoxia has been implicated in the pathogenesis of fibrosis and proximal tubular epithelial cells (PTE) are sensitive to oxygen deprivation, we hypothesized that hypoxia may stimulate ECM accumulation. In human PTE, hypoxia (1% O_2, 24 hr) increased total collagen production (15%), decreased MMP-2 activity (55% ± 13%; control = 100%) and increased tissue inhibitor of metalloproteinase-1 (TIMP-1) protein. Collagen IV mRNA levels decreased while collagen I mRNA increased, suggesting induction of interstitial collagen. Hypoxia-induced changes persisted on re-oxygenation with increased expression of TIMP mRNAs. A potential mediator for these effects is transforming growth factor-β_1 (TGF-β_1), a major pro-fibrogenic factor produced by PTE. Although hypoxia stimulated TGF-β production (2- to 3-fold), neutralizing anti-TGF-β_1 antibody did not abolish the hypoxia-induced changes in gelatinase activity, TIMP-1, collagen IV or collagen I mRNA expression, implying that TGF-β_1 is not the mediator. Furthermore, exogenous TGF-β_1 (0 to 10 ng/ml) did not mimic hypoxia, as it stimulated MMP-2 activity and increased the expression of collagen IV, collagen I and TIMP-1 mRNA. The data suggest that hypoxia may be an important pro-fibrogenic stimulus independent of TGF-β_1.

Progressive renal disease, characterized by accumulation of extracellular matrix (ECM) and expansion of the tubulointerstitial compartment, represents a significant clinical problem culminating in renal failure [1]. The mechanisms underlying the initiation and progression of fibrosis have not been identified. Among many pathogenetic mechanisms [2], we have suggested that primary injury to the proximal tubular cells (PTE) may lead to autocrine and paracrine changes in ECM metabolism and to alterations in interactions with adjacent cells (fibroblasts, capillary endothelia and infiltrating inflammatory cells), resulting in thickening of the tubular basement membrane (TBM) and the accumulation of matrix material within the interstitium [3]. One common pathological injury preceding the development of fibrosis is hypoxia [4–6]. Tubular cells may be subjected to low grade ischemia secondary to hypertensive injury to the microvasculature. Also, as ECM accumulates, physical separation of tubules and capillaries may reduce O_2 delivery and impair function. Further, pO_2 levels in the tubulo-interstitium (TI) are lower than systemic values rendering the TI relatively hypoxic, the increased metabolic activity of PTE attendant on increased filtered protein in the diseased kidney in combination with reduced O_2 availability may lead to ischemia of the tubular epithelia. Sensitivity of PTE to hypoxia [2, 7–9] is illustrated by isolated tubule models of ischemia/reperfusion in which oxygen deprivation leads to impaired protein synthesis [10], altered membrane transport [11, 12] and loss in cell polarity [13], suggesting these cells as the primary target for hypoxic injury. Expression of a number of genes have been shown to be altered by reduced oxygen [14, 15] and in different cell types, exposure to low oxygen tension induces expression of a variety of growth factors and cytokines [14–16], including several fibrogenic factors such as TGF-β [14, 17], PDGF [7] and endothelin-1 [18, 19]. Hypoxia can directly regulate the expression of these genes via oxygen-responsive DNA sequences, as first described in the regulation of the erythropoetin (EPO) gene [20, 21].

In addition to regulating cytokine gene expression, hypoxia is known to regulate matrix production in a number of cell systems [22–24], and although there are limited data available on the effects of hypoxia on matrix synthesis in the kidney, an increase in collagen gene expression has been demonstrated in mesangial cells under hypoxic conditions [25]. ECM accumulation occurs as a result of increased synthesis and/or decreased degradation [26], however, the effects of hypoxia on the degradative aspect of matrix metabolism have not been studied. ECM degradation is regulated by the matrix metalloproteinases (MMPs), a family of zinc-containing proteases that includes MMP-2 (gelatinase-A), MMP-9 (gelatinase-B), collagenases and stromelysins [27, 28]. MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs), a family of four inhibitors [29]. Previous studies from our group have shown that PTE produce MMP-2 and MMP-9 together with TIMP-1 and -2, and it is known that in fibrosis levels of MMP are either suppressed or unchanged while inhibitor levels are often increased [26, 30, 31]. Given that hypoxia may be a common component of renal fibrosis and a stimulus for increased matrix production in some cell types, we wished to establish whether hypoxia, either directly or indirectly, alters matrix turnover in renal PTE, thereby initiating events that lead to the fibrotic phenotype.

This study demonstrates that hypoxia simultaneously stimulates collagen production in PTE while decreasing the activities of MMP-2 and MMP-9 and increasing the expression of TIMPs,

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effects which appear to be largely independent of TGF-β_1. In addition to affecting tubular cell function, changes in matrix composition and turnover *in vivo* could alter paracrine interactions with neighboring fibroblasts, thereby initiating the cascade of events leading to fibrosis. Hypoxia-induced alterations in matrix metabolism may be common to diverse forms of organ fibrosis in which there is microvascular compromise and decreased tissue oxygen delivery.

METHODS

Cell culture

Human proximal tubular epithelial cells (PTE) were derived from the histologically normal pole of kidneys removed for carcinoma. Tissue was minced, passed through a 180 μm sieve (BDH Merck Ltd, Lutterworth, Leics, UK) to remove the glomeruli, plated on collagen I-coated tissue culture plastic (Greiner Labs, Dursley, Glos, UK) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), adenosine, guanosine, uridine, cytosine (12.5 mg/ml each), transferrin (5 μg/ml), insulin (5 μg/ml), dexamethasone (5×10^{-8} M), antibiotic/antimycotic (penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (25 ng/ml), HEPES (10 mM) (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 to 4 days after plating. Cells had a cobblestone morphology characteristic of epithelial cells, and cellular identity was confirmed by alkaline phosphatase activity and immunocytochemistry. The medium was changed every 3 to 4 days and cells were passaged at confluence using trypsin-EDTA (trypsin 0.5 mg/ml, EDTA 0.2 mg/ml; Gibco-BRL). Cells were used for experiments between passages 1 to 6. Confluent cells were made quiescent by 24 hour incubation in serum-free RPMI 1640 medium containing nucleosides, HEPES, transferrin and antibiotic/antimycotic (quiescence media).

Cell characterization

Assay for alkaline phosphatase activity. Alkaline phosphatase activity was measured using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) as described previously [32].

Immunocytochemistry. Cells at passage 2 (P2) were plated onto collagen I-coated 8-well chamber slides (Gibco-BRL), grown to semi-confluence (approximately 80% cover) and made quiescent. Cells were fixed with ice-cold methanol at −20°C and stained as described by Lewis, Fine and Norman [32] using the following primary antibodies diluted in 2% bovine serum albumin (BSA) in PBS: monoclonal anti-cytokeratin (CAM 5.2, 1:100 dilution; Becton Dickinson Cowley, Oxford, UK), monoclonal anti-vimentin (clone Vim 3B4, 1:250; Boehringer Mannheim), monoclonal anti-α-smooth muscle actin (1:1000; Sigma), monoclonal anti-desmin (1:1000; Sigma). Positive staining was detected using biotinylated secondary antibodies (Vectastain Elite ABC Kit, Vector Labs, Bretton, Peterborough, UK) followed by avidin-biotin complex (ABC). Positive reactivity was visualized using the chromogenic substrate aminoethylcarbazole (AEC-Substrate Kit, Vector Labs) giving a red-brown precipitate. Cells were counterstained with Harris' hematoxylin (Sigma) for 30 seconds 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).

Hypoxic conditions

PTE were made quiescent for 24 hours as described above. Fresh quiescence medium was added and cells incubated in 1% O_2, 5% CO_2, 94% N_2 in a Billups-Rothenberg Chamber™ (ICN-Flow, High Wycombe, Bucks, UK) for 24 hours. The oxygen concentration inside the chamber was confirmed at the end of the hypoxic period using a Radiometer ABL4 (Radiometer, Copenhagen, Denmark). The media was replaced with fresh quiescence media and the cells transferred to normoxia (21% O_2) for 24 hours (referred to as 'post-hypoxia'). Conditioned medium (CM) was collected at the end of hypoxia and at the end of the post-hypoxia period. Media were clarified by centrifugation at 1,000 × g for 15 minutes at 4°C and stored at −80°C prior to use. Total protein content was measured using a modified Bradford assay [32] on a DU-64 Spectrophotometer using the Soft-Pac™ Protein Assay Module (Beckman Instruments Ltd., High Wycombe, Bucks, UK). Protein concentration was determined by comparison to BSA standards (0 to 20 μg/ml) prepared in quiescence medium.

Measurement of cell viability

Cell viability was measured using both Trypan Blue and LDH release. For the Trypan Blue assay, aliquots of cell suspension were mixed with Trypan Blue (Sigma) in a 1:1 ratio and the number of dead cells (uptake of blue dye) were calculated as a percentage of the total cell number counted with a hemocytometer (Marathon Laboratory Supplies, London, UK). LDH release (as a percentage of total LDH) was measured using an LDH Assay Kit (Sigma) according to the manufacturer's instructions.

Measurement of cell number and cell proliferation

Confluent, quiescent PTE were incubated in 2 μCi/ml ^{3}H-thymidine (^{3}H-TdR; 26Ci/mmol; Amersham International plc, Little Chalfont, Bucks, UK) for two hours prior to harvest. Cells were washed with PBS and TCA-precipitable material was solubilized with 0.5 M NaOH. Radioactivity was counted in a Packard 2000CA Tri-Carb Liquid Scintillation Analyser (Canberra Packard, Pangbourne, Berks, UK) and calculated as disintegrations per minute (dpm) per well. Cell number was counted using a hemocytometer (Marathon).

Collagen production

Total collagen production was measured by reverse phase HPLC (System Gold, Beckman Instruments Ltd) [33]. Briefly, PTE were grown in 12-well plates (6 replicate wells/treatment) in RPMI 1640 media containing all supplements. At confluence, cells were transferred to incubation media [RPMI 1640 containing nucleosides, HEPES, transferrin, antibiotic/antimycotic, 23 μg/ml proline (BDH Merck) and 50 μg/ml ascorbate (Sigma)] for 24 hours. Fresh incubation medium was added to the cells prior to hypoxia (or exposure to control conditions). Cells were harvested after 24 hours hypoxia or after a further 24 hours post-hypoxia and the plates stored at −20°C prior to analysis. For HPLC, plates were thawed, the cell layer scraped into the media and the contents of each well collected. Wells were washed with PBS and the rinse solution added to the cell suspension. Proteins were

precipitated by addition of ethanol (to a final concentration of 67%). The samples were filtered through a 0.4 μm filter (type HV; Millipore, Watford, UK) and the filter plus proteins hydrolyzed in 6 M HCl at 110°C for 16 hours. The hydroxyproline content of the hydrolysates was assessed after separation of the 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) derivatives 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. Procollagen production was expressed as nM OH-proline/well.

Plates used for HPLC analysis of hydroxyproline were also used to quantify non-collagen protein synthesis by measurement of ^{3}H-phenylalanine (26 Ci/mmol; Amersham) in the hydrolysates. Wells were labeled with 5 μCi/ml ^{3}H-phenylalanine at the beginning of the 24 hours of hypoxia (for 24 hr) or immediately before the 24-hour post-hypoxia period (for 24 hr). Cells were processed as for HPLC and radioactivity was measured in aliquots of hydrolysate. Data are presented as dpm/well.

RNA extraction and Northern blot analysis

Total cellular RNA was extracted using TRIZOL^R Reagent (Gibco-BRL) according to the manufacturer's instructions. The concentration of RNA was measured by absorbance at 260 nm and samples were stored at −80°C prior to use. For Northern blot analysis, 10 μg of total RNA were electrophoresed on a 1.2% agarose (ICN Flow) gel, containing 1.85% formaldehyde using 1X MOPS pH 7.0 (20 mM MOPS (3-[N-morpholino]propanesulfonic acid), 5 mM sodium acetate, 1 mM EDTA) 3.7% formaldehyde as the running buffer. Ethidium-bromide-stained gels were photographed under UV illumination. RNA was capillary blotted to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 16 to 18 hours with 1 M ammonium acetate as the transfer buffer and blots baked under vacuum at 80°C for two hours. Membranes were prehybridized overnight at 65°C in 4 × SSC (0.6 M NaCl, 60 mM Na_3citrate·$2H_2O$), 5× Denhardt's solution [4 mg/ml polyvinylpyrrolidone (PVP), 4 mg/ml BSA, 4 mg/ml Ficoll; Sigma Chemical Company] 0.5% SDS, 5 mM Tris, 1 mM EDTA in a Techne Hybridization Oven (Techne Ltd, Duxford, Cambs, UK). Blots were hybridized overnight at 65°C in prehybridization solution containing 50 μg/ml pA^+-RNA (Boehringer Mannheim), 50 μg/ml denatured salmon sperm DNA (Sigma) and 10^7 dpm of α^{32}P-dCTP-labeled cDNA probe. Blots were washed 4 times, 20 minutes each, in 4 × SSC, 0.5% SDS at 50°C and exposed to autoradiography film (Kodak XAR; KJP, London, UK) at −80°C with intensifying screens for 1 to 6 days. Photographs of ethidium bromide-stained gels and autoradiograms were scanned and quantified by densitometry (ImageMaster Software; Pharmacia, St Albans, Herts, UK). Values were corrected for variations in RNA loading by comparison to densitometry values of ethidium bromide-staining of rRNA normalized to control. Data are expressed as relative mRNA levels calculated as a percentage of the relevant control value (assigned a value of 100%) from arbitrary densitometry units.

Probes

cDNA probes for human MMP-2, TIMP-1 and TIMP-2 were a generous gift from Dr. G. Murphy (Strangeways Laboratory, Cambridge, UK). 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). Probes were labeled with α^{32}P-dCTP (3000Ci/mmol; Amersham) using a Random Primed DNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions.

MMP-2 and MMP-9 (gelatinases) activity

Gelatinase activity in CM was measured by gelatin-substrate gel zymography [32]. Protein concentration of conditioned media was measured using a modified Bradford assay [32] and equal amounts of protein were loaded following concentration with Microcon-3 (Amicon Ltd, Stone House, Glos, UK) with a molecular weight cut off at 3 kDa. Zymography was performed as described by Lewis et al [32]. Gels were scanned and bands quantified by densitometry (ImageMaster Software; Pharmacia). Relative MMP-2 activity was calculated as a percentage of the relevant control value (assigned the value of 100%) from arbitrary densitometry units.

ELISA for TIMP-1

TIMP-1 protein in conditioned media was measured using the Biotrak Human TIMP-1 ELISA System (Amersham) according to the manufacturer's instructions. Briefly, 100 μl aliquots of standards (0 to 50 ng/ml TIMP-1) or conditioned media samples [diluted 1:5 in assay buffer (Amersham)] were incubated in 96-well plates pre-coated with anti-TIMP-1 antibody. Bound TIMP-1 was detected using a peroxidase-labeled anti-TIMP-1 antibody that produces a blue color reaction on addition of the substrate 3,3′ 5,5′-tetramethylbenzidine (TMB). The addition of 1 M H_2SO_4 produced a yellow color change, absorbance was read at 450 nm using a Multiskan MCC/340 plate reader (Titertek, Labsystems, Finland). The concentration of TIMP-1 in CM was determined from the standard curve.

Bioassay for TGF-β

The mink lung epithelial cell line (CCL64; ECACC, Salisbury, Wilts, UK) bioassay [34] was used to measure levels of latent/active TGF-β (all isoforms) in PTE-CM. CCL64 are growth inhibited by TGF-β, hence the degree of ^{3}H-TdR incorporation in response to TGF-β_1 standards can be used to quantify TGF-β (all isoforms) in CM. Latent TGF-β, produced by PTE, was activated by heating CM samples at 80°C for 10 minutes prior to addition to CCL64. Briefly, CCL64 were seeded at a density of 5×10^{-3}/well in Dulbecco's Modified Eagle Medium F12 (DMEM/F12, 1:1 mixture; Gibco-BRL) containing 10%FCS and incubated for 24 hours, the serum level was then reduced to 1% for a further 24 hours. Conditioned media (equal volume to existing media) was added to each well for 24 hours and the cells labeled with 1 μCi/well of ^{3}H-TdR (Amersham) for five hours. Protein and DNA were then precipitated, solubilized and assayed as in the proliferation assay. Concentrations of TGF-β in CM were measured by comparison to a standard curve of exogenously added TGF-β_1 (0 to 10 ng/ml; Genzyme Diagnostics, West Malling, Kent, UK) prepared in serum-free RPMI media. Data are expressed as ng/ml active and total TGF-β in CM from PTE exposed to 24 hours hypoxia or at 24 hours post-hypoxia with respect to the relevant controls.

Effect of exogenous TGF-β_1 on PTE

TGF-β_1 (2.5 ng/ml or 10 ng/ml; Genzyme Diagnostics) was added to cells under normoxic conditions for 24 hours. mRNA

levels of MMP-2, TIMP-1, collagen I and collagen IV were measured by Northern blot analysis and quantified by densitometry as described above. To investigate the effect of exogenous TGF-β_1 on MMP-2 activity, confluent, quiescent PTE were treated with 0 to 10 ng/ml TGF-β_1 for 24 hours under normoxic conditions. CM was collected, clarified and analyzed by gelatin-substrate gel zymography. Zymograms were scanned and quantified by densitometry as described above. Data are expressed as relative MMP-2 activity calculated as a percentage of the relevant control (assigned the value of 100%) from arbitrary densitometry units.

Neutralization of TGF-β_1 activity

To determine whether the effects of hypoxia on PTE are mediated by TGF-β_1, anti-TGF-β_1 antibody (chicken IgY, 1.2 μg/ml or 10 μg/ml; R&D Systems Europe Ltd, Abingdon, Oxon, UK) was added to cells immediately before hypoxia and at the beginning of the post-hypoxia period. Parallel experiments were undertaken with non-immune chicken IgG (Sigma) added at the same concentrations as the neutralising TGF-β antibody to examine the specificity of the blocking effect. The efficacy of the antibody was tested by simultaneous addition of antibody and exogenous TGF-β_1 (10 ng/ml; Genzyme Diagnostics) to PTE under normoxic conditions. MMP-2 activity was assessed by quantitation of gelatin-substrate gel zymography as above and relative MMP-2 activity calculated as a percentage of the relevant control (assigned a value of 100%) from arbitrary densitometry units). Levels of mRNA expression of MMP-2, TIMP-1, collagen I and collagen IV were measured by Northern blot analysis and quantitated as described above.

Statistical analysis

Data were analyzed 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 were calculated, standard errors of the mean are also given.

RESULTS

Characterization of human PTE

Cellular outgrowth from attached cortical tissue explants generally had a cobblestone appearance characteristic of epithelial cells. Cells were positive for alkaline phosphatase (> 90%) suggesting that cultures were composed predominantly of PTE [32]. By immunocytochemistry, cells 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 characteristsic of myofibroblasts and mesangial cells). Taken together, these data confirmed the outgrowing cell population to be predominantly epithelial. For experiments, cells were used up to passage 6, as immunocytochemistry of cells at higher passage numbers showed some positive staining for vimentin, suggesting a possible de-differentiation with repeated passaging *in vitro*.

Effects of hypoxia on viability and cell proliferation

Cells were examined after 24 hours hypoxia and 24 hours post-hypoxia for qualitative changes. Hypoxia had no effect on cell morphology. There was no significant difference in cell number per 10 cm^2 dish (control, $3.27 \times 10^6 \pm 0.35 \times 10^6$ cells vs. hypoxia, $3.30 \times 10^6 \pm 0.46 \times 10^6$) or in cell viability as measured by Trypan Blue-exclusion (number of control cells stained blue, $1.55 \times 10^4 \pm 0.10 \times 10^4$ vs. hypoxic cells, $1.41 \times 10^4 \pm 0.12 \times 10^4$). Measurement of LDH released into the media calculated as a percentage of total LDH confirmed that hypoxia had no effect on cell viability (control, 44.9% ± 2.2% vs. hypoxia, 45.5% ± 0.8%). Furthermore, ^{3}H-TdR incorporation showed that there was no effect on cell proliferation either at the end of hypoxia or after the post-hypoxia period (Fig. 1). These data suggest that exposure to 1% O_2 for 24 hours does not cause macroscopic injury or cell death, but cells showed increased attachment to the substrate with treated cells requiring more prolonged trypsinization at passage.

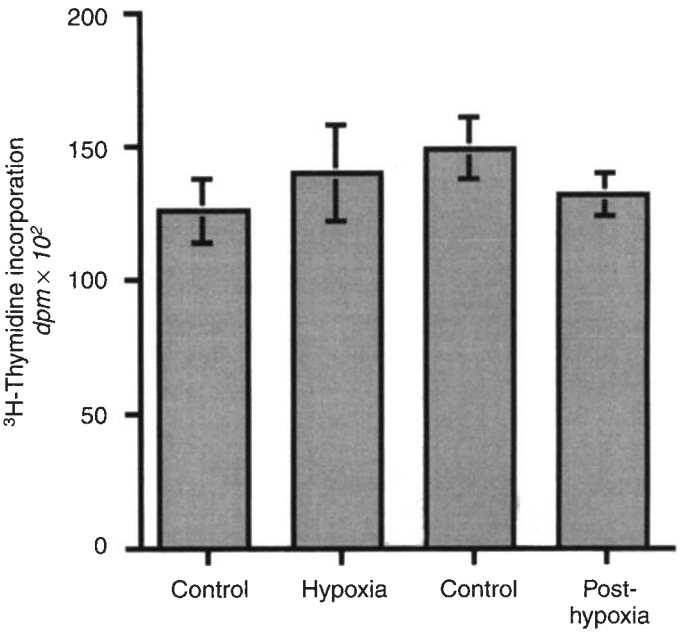

Fig. 1. ^{3}H-thymidine incorporation of PTE exposed to 24 hours of hypoxia and 24 hours post-hypoxia. Confluent, quiescent cells were labeled with 2 μCi/ml of ^{3}H-TdR for two hours prior to harvest after 24 hours normoxia (Control), 24 hours hypoxia (Hypoxia), 48 hours normoxia (Control), and 24 hours post-hypoxia (Post Hypoxia). ^{3}H-TdR incorporation is expressed as disintegrations per min/well (dpm/well). Data are the mean (± SEM) of 3 experiments with $N = 6$ wells in each sample/experiment.

Effect of hypoxia on collagen production and gene expression

Collagen production was measured by HPLC of cellular and secreted hydroxyproline. Cells plus media were collected after 24 hours hypoxia and at 24 hours post-hypoxia. Hydroxyproline levels were increased after hypoxia (Fig. 2A) and continued to increase post-hypoxia with respect to the relevant control, achieving statistical significance 24 hours post-hypoxia ($P < 0.05$). To establish whether this effect was specific for collagen, ^{3}H-phenylalanine incorporation into total protein was examined. As shown in Fig. 2B, total protein synthesis was decreased after 24 hours of hypoxia and remained suppressed in the post-hypoxia period, indicating that the stimulatory effect of hypoxia on collagen production may be selective for specific proteins.

Northern blot analysis was performed to establish whether

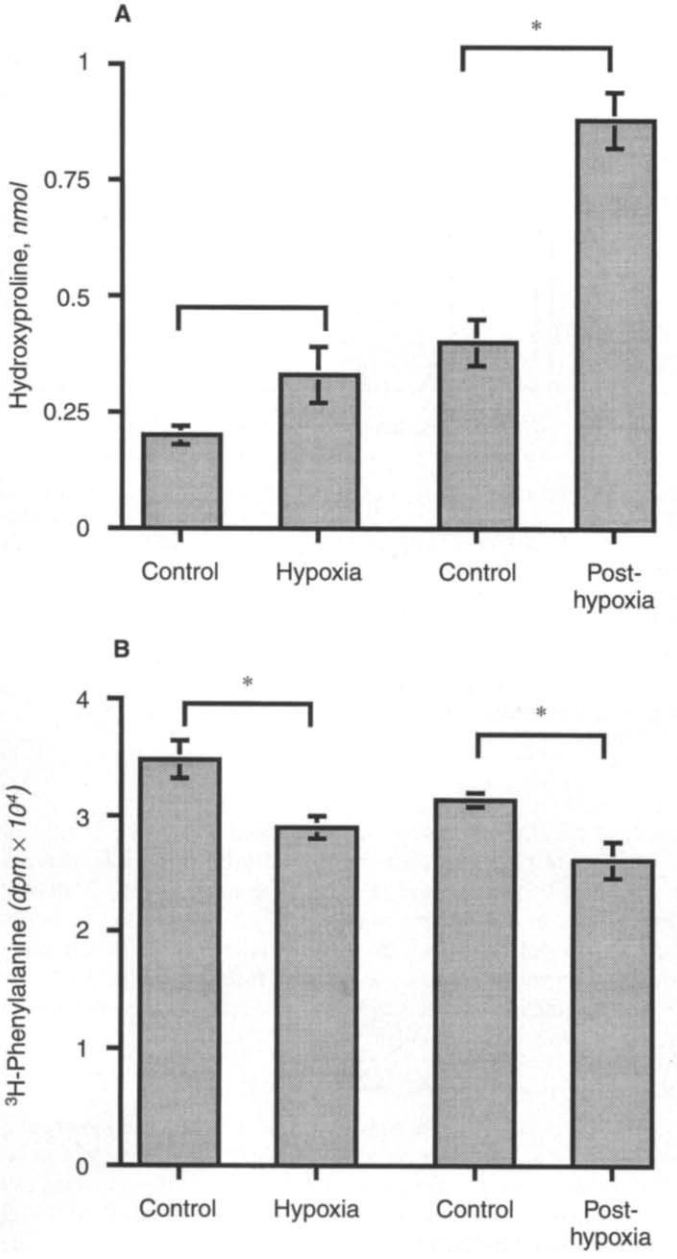

Fig. 2. *A.* Collagen production by PTE exposed to 24 hours of hypoxia or 24 hours post-hypoxia. Hydroxyproline levels in confluent and quiescent PTE exposed to 24 hours of normoxia (21% O_2, Control), 24 hours of hypoxia (1% O_2, Hypoxia), 48 hours of normoxia (Control), and 24 hours post-hypoxia (Post-hypoxia) were measured by HPLC analysis of cells plus media. Concentrations of sample hydroxyproline were calculated from a HPLC standard: 1 HPLC unit = 1 nm hydroxyproline. Data are the mean of 2 experiments ± SEM, $N = 6$ wells in each sample/experiment. $^*P < 0.05$ by unpaired Student's *t*-test. (*B*) ^{3}H-phenylalanine incorporation of PTE exposed to 24 hours hypoxia or in the period post-hypoxia. PTE used for HPLC were labeled with: ^{3}H-phenylalanine 24 hours normoxia, 21% O_2 (Control); 24 hours hypoxia, 1% O_2 (Hypoxia); 48 hours normoxia (Control); 24 hours post-hypoxia (Post Hypoxia). Data are the mean of 2 experiments with $N = 6$ wells in each sample, $^*P < 0.05$ by unpaired Student's *t*-test.

increased collagen production was due to increased gene expression. Collagen type IV is the major constituent of the PTE basement membrane, however, collagen IV mRNA levels (Fig. 3)

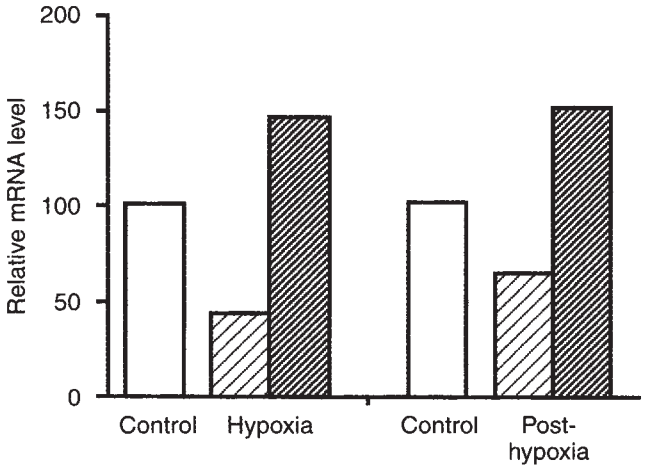

Fig. 3. Northern blot analysis of collagen IV (▨) and collagen I (▨) mRNA expression of PTE after 24 hours hypoxia and 24 hours post-hypoxia. Total RNA was extracted from PTE exposed to: 24 hours normoxia 21% O_2 (Control); 24 hours hypoxia 1% O_2 (Hypoxia); 48 hours normoxia (Control); 24 hours post-hypoxia (Post Hypoxia). Autoradiograms were scanned and quantified by densitometry. Values were corrected for variations in RNA loading by comparison to densitometry values of ethidium bromide staining of rRNA. Relative mRNA levels were calculated as a percentage of the relevant control values [assigned 100% values (□)]. The data presented are a representative experiment of 3 repeats.

decreased, rather than increased, after 24 hours of hypoxia (46% of control) and remained suppressed post-hypoxia. In contrast, expression of mRNA for the interstitial collagen, collagen I, was stimulated by hypoxia. PTE expressed low levels of collagen I mRNA that increased by 48% after 24 hours of hypoxia and remained elevated post-hypoxia (Fig. 3).

Effect of hypoxia on gelatinase gene expression and activity

Gelatin-substrate gel zymography generally revealed two major bands of activity in CM from PTE maintained in normoxia for 24 hours; one band at ~72 kDa representing MMP-2 (gelatinase A) and another at ~92 kDa representing MMP-9 (gelatinase B). Both bands often 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. MMP-2 was observed in all PTE-CM while the appearance of MMP-9 was more variable. After 24 hours of hypoxia, there was a significant decrease in MMP-2 activity (55% ± 13% of control value, assigned 100%; Fig. 4A), which remained suppressed on return to normoxia for 24 hours (65% ± 13.4% of the respective control). The decrease in MMP-2 activity on treatment with hypoxia is independent of passage number with a variable decrease observed at all passage numbers. MMP-9 showed a similar pattern of activity (data not shown). Northern blot analysis demonstrated that MMP-2 mRNA levels were not affected by hypoxia (Fig. 4B), suggesting that the decrease in MMP-2 activity may be the result of post-transcriptional regulation.

Effect of hypoxia on TIMP mRNA and protein

To evaluate hypoxia-induced changes in enzyme inhibitors, TIMP-1 and TIMP-2 expression was examined. Northern blot

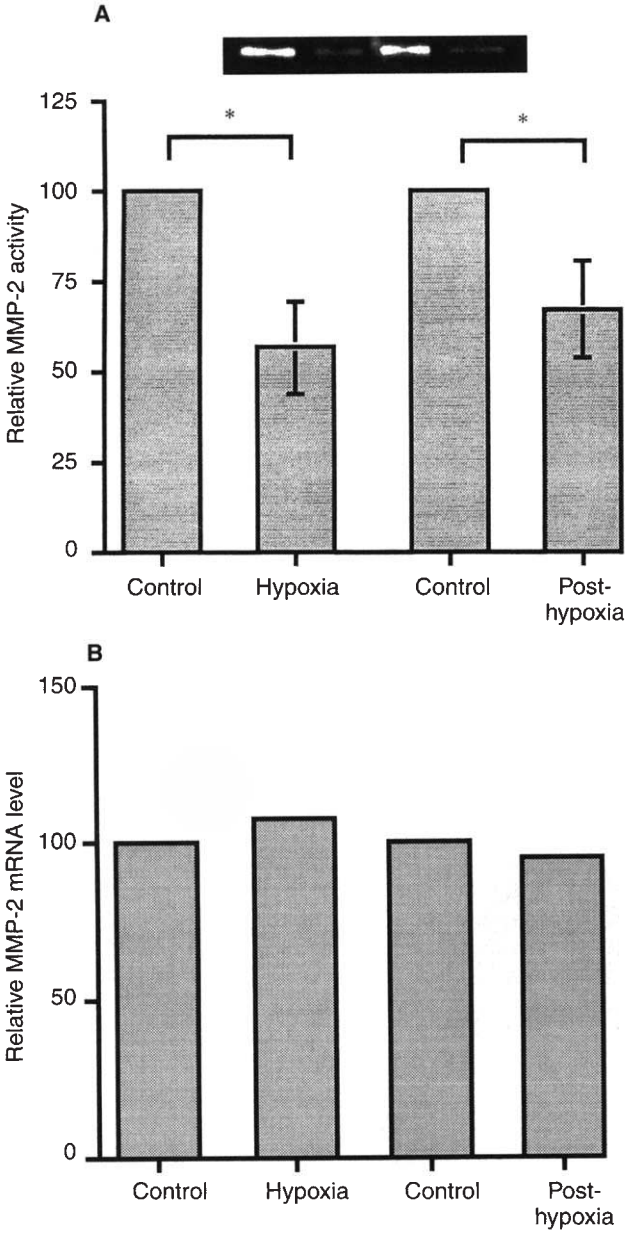

Fig. 4. *A.* Gelatin-substrate gel zymography of the effect of hypoxia on MMP-2 in PTE. Conditioned media was collected from confluent, quiescent PTE exposed to 24 hours normoxia, 21% O_2 (Control); 24 hours hypoxia, 1% O_2 (Hypoxia); 48 hours normoxia (Control); 24 hours post-hypoxia (Post Hypoxia). CM was concentrated and equal protein electrophoresed per lane. Gels were scanned and quantified by densitometry and relative MMP-2 activity was calculated as a percentage of the relevant control values (assigned values of 100%) from arbitrary densitometry units. Data are the mean of 3 experiments. Results were analyzed by unpaired Student's *t*-test, $P < 0.05$. **Insert**: Photograph of a representative zymogram with lane order as in the graph. (*B*) Northern blot analysis of MMP-2 mRNA expression of PTE after 24 hours of hypoxia and 24 hours post-hypoxia. Total RNA was extracted from PTE exposed to: 24 hours normoxia 21% O_2 (Control); 24 hours hypoxia 1% O_2 (Hypoxia); 48 hours normoxia (Control); 24 hours hypoxia post-hypoxia (Post Hypoxia). Autoradiograms were scanned and quantified by densitometry. Values were corrected for variations in RNA loading by comparison to densitometry values of ethidium bromide staining of rRNA. Relative mRNA levels were calculated as a percentage of the relevant control values (assigned values of 100%) from arbitrary densitometry units. Data shown are a representative experiment of 3 repeats.

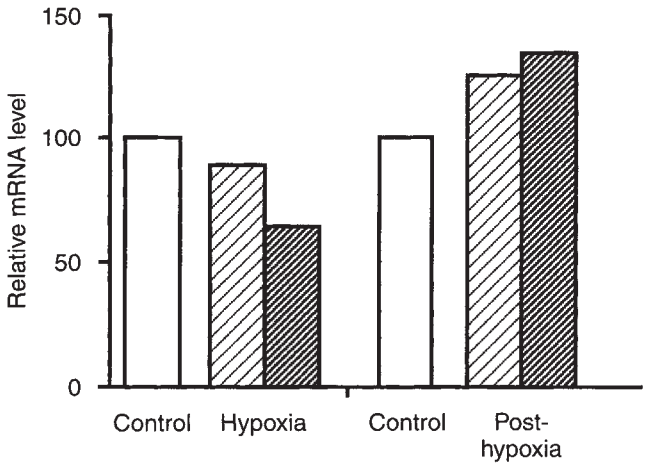

Fig. 5. Northern blot analysis of TIMP-1 (▨) and TIMP-2 (▨) mRNA. Total RNA was extracted from PTE exposed to 24 hours normoxia 21% O_2 (Control); 24 hours hypoxia 1% O_2 (Hypoxia); 48 hours normoxia (Control); 24 hours post-hypoxia (Post Hypoxia). Autoradiograms were scanned and quantified by densitometry. Values were corrected for variations in RNA loading by comparison to densitometric values of ethidium bromide-stained rRNA. Relative mRNA levels were calculated as a percentage of the relevant control values (□, assigned the values of 100%) from arbitrary densitometry units. Data are a representative experiment of 2 to 3 repeats.

analysis of TIMP-1 mRNA levels showed this to be similar in 24-hour control and hypoxia-treated cultures, with some increase in the post-hypoxia period (Fig. 5). In contrast, TIMP-2 mRNA levels decreased by approximately 35% of control after 24 hours of hypoxia but then rose above the control value in the post-hypoxia period (Fig. 5). Secreted TIMP-1 protein levels in PTE-CM (measured by ELISA) increased after 24 hours hypoxia (20% above control; data not shown).

Effect of hypoxia on expression of TGF-β by PTE

TGF-β_1 is the major fibrogenic cytokine within the kidney and is induced by hypoxia in a variety of cell types. Expression of TGF-β by PTE was examined using the mink lung epithelial cell line CCL64 bioassay to measure active and total TGF-β (all isoforms) in PTE-CM [35]. CCL64 cells are growth inhibited by TGF-β such that the degree of ^{3}H-TdR incorporation in response to known concentrations of exogenous TGF-β_1 can be compared with inhibition by conditioned media samples. Hypoxia induced a 160% increase in the amount of active TGF-β: 1 ± 0.09 ng/ml in control versus 2.6 ± 0.18 ng/ml after 24 hours of hypoxia (Fig. 6A). Levels of total TGF-β in CM (active and latent) were also increased following hypoxia: 9.5 ± 0.92 ng/ml versus 3.0 ± 0.47 ng/ml in control cultures (Fig. 6B). Assay of CM from cells in the 24-hour post-hypoxia period showed a decrease in both active TGF-β (1.2 ± 0.09 ng/ml vs. 4.0 ± 0.2 ng/ml in control) and total TGF-β (6.5 ± 0.41 ng/ml vs. 8.0 ± 0.82 ng/ml in control).

Effect of exogenous TGF-β_1 on MMP-2 mRNA and activity, TIMP-1, collagen I and collagen IV mRNA expression

Since TGF-β_1 is known to regulate matrix synthesis and turnover in a number of cell types [27], experiments were undertaken to establish the effect of this factor on matrix metabolism. PTE

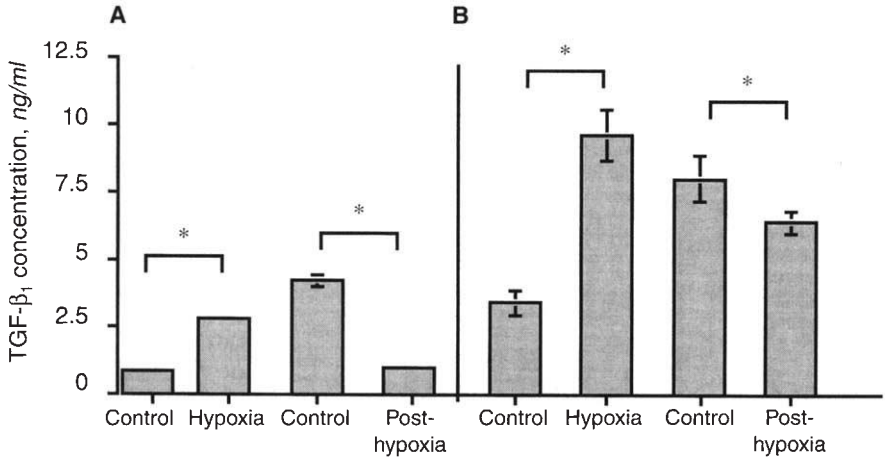

Fig. 6. Effect of hypoxia on levels of (*A*) active and (*B*) total TGF-β in PTE-CM. TGF-β in CM from PTE were measured using the CCL64 bioassay: CM from control cells maintained for 24 hours in normoxia (Control); CM from control cells maintained for 24 hours in hypoxia (Hypoxia); CM collected from cells exposed to normoxia for 48 hours (Control); CM from PTE exposed to 24 hours post-hypoxia (Post Hypoxia). TGF-β concentrations in CM (sample number, $N = 6$ wells) were calculated from a TGF-β_1 standard curve (not shown). Data shown is a representative experiment of 3 repeats where all results were significant, $^*P < 0.05$, unpaired Student's t-test.

were treated with 0 to 10 ng/ml TGF-β_1 under normoxic conditions for 24 hours, cells were harvested for RNA and CM collected for zymography. MMP-2 activity increased in response to TGF-β_1 in a dose-dependent manner (Fig. 7A). To examine the effect of exogenous TGF-β_1 on mRNA expression of MMP-2, TIMP-1, collagen I and collagen IV, two concentrations of TGF-β_1 were tested: (1) 2.6 ng/ml, equivalent to the concentration of active cytokine produced by hypoxia-induced PTE as calculated from the bioassay data (Fig. 6); and (2) 10 ng/ml, equivalent to total TGF-β produced by PTE in hypoxia (Fig. 6). By Northern blotting, TGF-β_1 (2.5 ng/ml) stimulated MMP-2, TIMP-1, collagen I and collagen IV mRNA levels (data not shown). Addition of exogenous TGF-β_1 at 10 ng/ml further stimulated expression of all mRNAs (Fig. 7B), demonstrating a dose-dependent effect of TGF-β_1.

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

To establish whether the hypoxia-induced effects are mediated by TGF-β_1, PTE were exposed to hypoxia in the presence of two concentrations of neutralising anti-TGF-β_1 antibody: 1.2 μg/ml, sufficient antibody to block all of the active TGF-β produced by the cells after 24 hours hypoxia (2.6 ng/ml, calculated from TGF-β bioassay data; Fig. 6); and 10 μg/ml, sufficient to neutralize total TGF-β produced by PTE under hypoxic conditions (active plus latent, 9.5 ng/ml; Fig. 6). In PTE treated with TGF-β_1 (10 ng/ml), 1.2 μg/ml anti-TGF-β_1 antibody blocked the TGF-β_1-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%). 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 concentrations equivalent to the neutralizing antibody; non-immune IgG had no effect on TGF-β-induced increases in MMP-2 expression and collagen I mRNA levels. Although the anti-TGF-β_1 antibody had some small effect, neither concentration eliminated the hypoxia-induced decrease in MMP-2 activity (Fig. 8) or the increase in collagen I mRNA expression (Fig. 9) and collagen IV, MMP-2 or TIMP-1 mRNA levels (data not shown). The control IgG had no effect on hypoxia-induced changes.

DISCUSSION

The mechanisms underlying the initiation and progression of fibrosis have not been identified [35]. Since fibrotic renal diseases of diverse etiology are commonly associated with vascular compromise [4–6], we hypothesized that tissue hypoxia, either alone or in conjunction with other factors, may be a pro-fibrogenic stimulus [6]. Although all cellular components of the tubulointerstitium (tubular epithelial cells, capillary endothelia and interstitial fibroblasts) are likely to be affected by decreased oxygen availability, the metabolic functions of PTE confer high oxygen demands [36, 37], rendering these cells particularly vulnerable to fluctuations in oxygen levels *in vivo* [2, 7–10] and leading to the suggestion that tubular cells may be the primary target of a hypoxic insult [3], setting in train a cascade of autocrine and/or paracrine changes that culminate in the accumulation of ECM within the tubulo-interstitium.

The period of hypoxia used in this study (24 hr, 1% O_2) was based on data showing that *in vitro* exposure of various cell lines to 1% O_2 for a minimum of 16 hours alters growth factor gene expression [14]. In PTE, 24 hours hypoxia had no macroscopic effects on cell morphology, attachment or viability, although there was some decrease in total protein synthesis consistent with the findings of other groups that hypoxia impairs PTE protein synthesis [10]. Of interest is the observation that PTE exposed to hypoxia adhered more strongly to the substrate, perhaps reflecting hypoxia-induced changes in expression of integrins and/or cell adhesion molecules as has been reported in other cell types [38, 39]. Although previous studies in our laboratory reported that 48 hours of hypoxia (1% O_2) stimulated PTE proliferation [40], which may be important in tubular regeneration after injury, the shorter period of hypoxia used here had no effect on proliferation, suggesting that the proliferative response may be secondary, due, for example, to the induction of a mitogenic growth factor [14–17].

In the normal kidney, the TBM, composed predominantly of collagens IV and V, laminins, nidogen, fibronectin, heparan sulphate proteoglycans and glycoproteins [41], separates epithelial cells from neighboring cell types [42] and is essential for cell

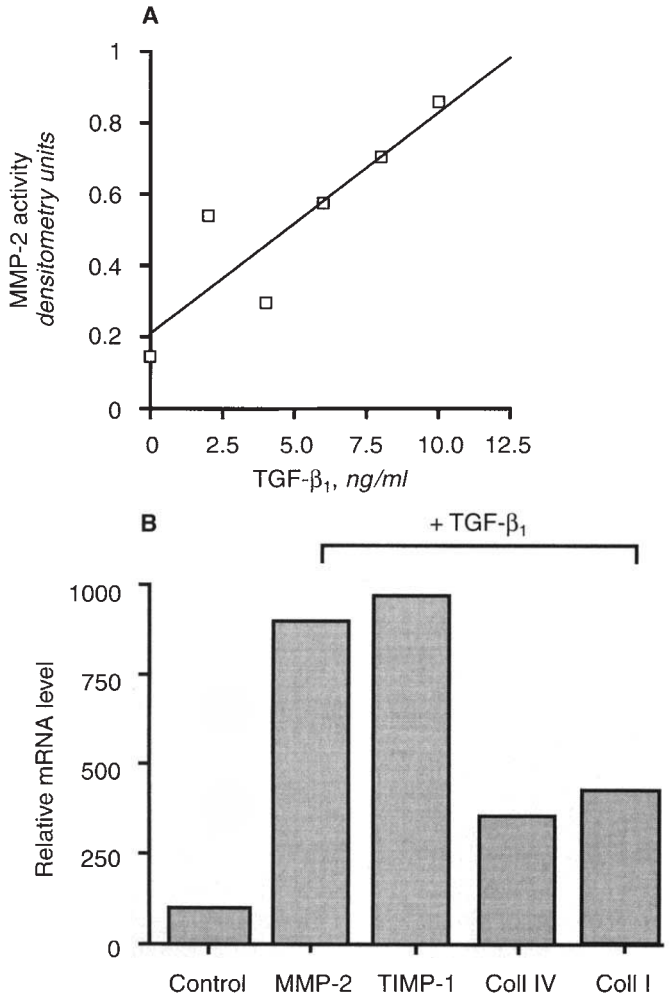

Fig. 7. *A.* Effect of exogenous TGF-β_1 on MMP-2 activity in normoxic PTE. Confluent, quiescent PTE were treated with 0 to 10 ng/ml TGF-β_1 for 24 hours. Gelatin-gel zymography was performed on conditioned media and MMP-2 activity quantified by densitometry (arbitrary units). The Figure shows a representative experiment of 3 repeats. (*B*) Northern blot analysis of MMP-2, TIMP-1, collagen I and collagen IV mRNA levels in normoxic PTE treated with exogenous TGF-β_1. RNA was extracted from confluent, quiescent PTE maintained in: normoxia for 24 hours (Control); normoxia for 24 hours in the presence of exogenous TGF-β_1 (10 ng/ml; +TGF-β_1). Autoradiograms were scanned and quantitated as above. Relative mRNA levels were calculated as a percentage of the control value (assigned the value of 100%) from arbitrary densitometry units. The data show a representative experiment of 3 repeats.

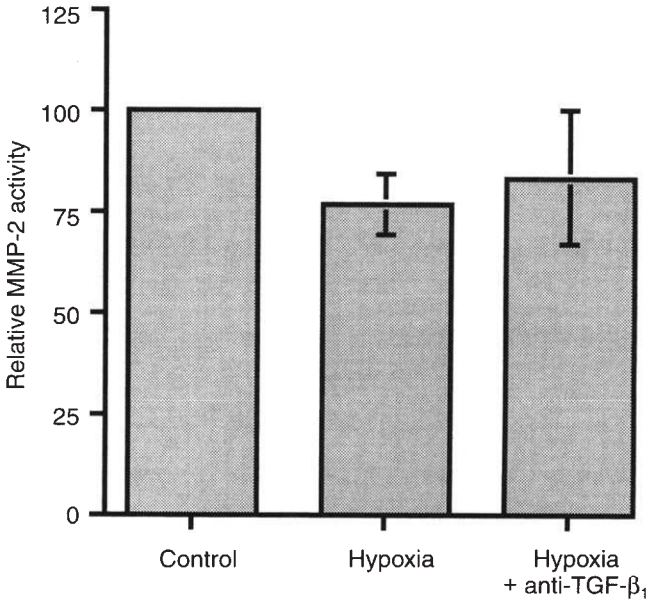

Fig. 8. Effect of anti-TGF-β_1 antibody on MMP-2 activity in PTE exposed to 24 hours hypoxia. CM was collected from PTE exposed to 24 hours normoxia (Control); 24 hours hypoxia (Hypoxia) or 24 hours hypoxia in the presence of anti-TGF-β_1 antibody (Hypoxia + anti-TGF-β_1). Gelatin-gel zymography was performed and quantified by densitometry. Relative MMP-2 activities were calculated as a percentage of the control value (assigned the value of 100%). Data shown are the mean of 4 experiments ± SEM.

function and interactions [32, 41]. The normal matrix is maintained by a balance between synthesis and turnover. In fibrosis, increased matrix synthesis and/or decreased degradation, leads to thickening of the TBM and the appearance of abnormal matrix proteins [1, 35]. The mediators of changes in ECM metabolism in fibrosis have not been identified, but hypoxia has been reported to stimulate matrix synthesis in other cell systems with increased expression of α1-procollagen, tropoelastin and fibronectin mRNAs [22–24]. Although the effects of hypoxia on ECM synthesis have not been extensively studied in the kidney, increased collagen gene expression has been reported in hypoxic mesangial cells [25]. Hypoxia (24 hr) markedly increased PTE collagen production which increased further on return to normoxia. This increase in total collagen production occurred despite an overall decrease in protein synthesis, suggesting differential effects of hypoxia on collagen and non-collagen proteins. Since collagen IV is the major collagen component of the TBM [41, 43], it was of interest to determine whether increased collagen production was due to an increase in collagen IV. Surprisingly, collagen IV mRNA levels decreased after hypoxia and remained suppressed even after restitution of normal oxygen concentrations. 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 prodution 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 [2, 35]. Since matrix proteins themselves act as regulators of cell growth, differentiation and function [44] the presence of atypical collagen within the BM will likely have profound effects on tubular cell behavior affecting both autocrine and paracrine functions. The increase in collagens type I and III in the fibrotic interstitium is largely attributed to interstitial fibroblasts; however, the increased expression of interstitial collagens by PTE raises the possibility that, in addition to expansion of the TBM, PTE may also contribute to interstitial matrix accumulation. In addition, hypoxia has been reported to affect the processing of collagen producing a weaker molecule [23]. It is interesting to speculate that similar changes in the TBM, which provides structural support for the tubular cells, might contribute to tubular collapse observed in the advanced fibrosis [1].

On the other side of the balance, the major physiological

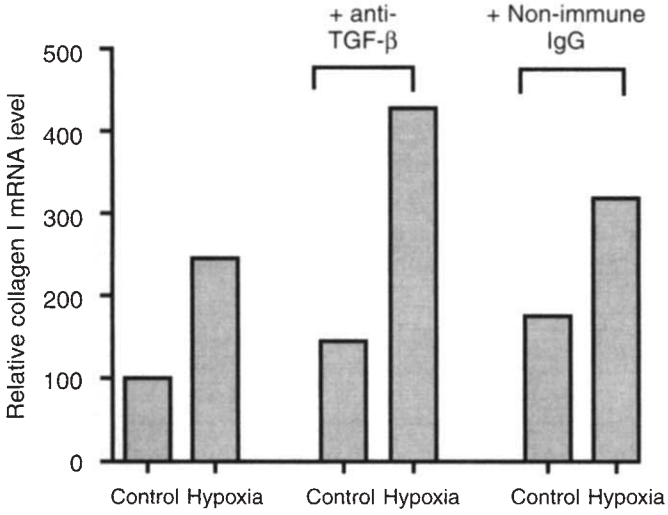

Fig. 9. Effect of anti-TGF-β_1 antibody on collagen I mRNA in PTE exposed to 24 hours of hypoxia compared to the addition of non-immune serum IgG. Total RNA was extracted from PTE exposed to: 24 hours of normoxia 21% O_2 (Control); 24 hours hypoxia 1% O_2 (Hypoxia); normoxia or hypoxia in the presence of 1.2 μg/ml anti-TGF-β_1 (+ anti-TGF-β); exposed to normoxia or hypoxia in the presence of 1.2 μg/ml non-immune serum IgG (+ non-immune serum IgG). Autoradiograms were scanned and quantitated as above. Relative mRNA levels were calculated as a percentage of the control value (assigned the value of 100%) from arbitrary densitometry units. The data show a representative experiment.

regulators of matrix turnover are the MMPs, with enzyme activity controlled by the TIMPs [27–29]. Renal cells express a variety of MMPs and TIMPs [26, 45] and changes in enzymes and/or inhibitor levels have been reported in a number of models of fibrosis and in human disease [30, 31, 46, 47], suggesting an important role for decreased degradation in the accumulation of ECM. Gelatinases are likely to be involved in BM turnover as their primary substrate is collagen IV, a major constituent of the BM [41, 43]. The data presented here demonstrate that, *in vitro*, hypoxia suppresses activity of both MMP-2 and MMP-9 in PTE cells by 45% and that this decrease is sustained when cells are returned to normoxia, a situation analogous to ischemia/reperfusion injury (although the decrease is not exacerbated as might be expected in reperfusion injury). Regulation of MMP-2 activity appears to be post-transcriptional since mRNA levels were not affected by hypoxia. Hypoxia-induced increases in mRNA stability have been reported in other systems [48], however, increased stability would be reflected in increased steady-state levels. Thus the data in PTE point to translational or post-translational regulation of enzyme activity. Although gelatinases can be inhibited by any of the TIMPs, the inhibitor most widely studied in the kidney is TIMP-1 [30, 31], which is expressed by normal PTE and interstitial fibroblasts [26, 36, 37, 47]. TIMP-2 is also expressed, albeit at lower levels (unpublished observations). In this study, secreted TIMP-1 was increased after 24 hours of hypoxia, but there was no change in mRNA expression suggesting post-transcriptional regulation of the inhibitor in this period. TIMP-2 mRNA levels were transiently suppressed after hypoxia, but both TIMP-1 and TIMP-2 mRNA levels increased on return to normoxia, implying a delayed increase in these inhibitors. These data suggest that hypoxia decreases PTE matrix degradation via

Table 1. Comparison of the effects of hypoxia and TGF-β_1

	Hypoxia	TGF-β_1
Collagen production	↑	ND
Collagen I mRNA	↑	↑
Collagen IV mRNA	↓	↑
MMP-2 mRNA	NC	↑
activity	↓	↑
TIMP-1 mRNA	(↑)	↑
protein	↑	ND
TIMP-2 mRNA	↑	ND

Abbreviations are: NC, no change; ND, no data.

changes in enzyme activity and inhibitor levels and, simultaneously, increases collagen production. Similar effects of decreased oxygen *in vivo* would lead to an accumulation of matrix and have repercussions on tubular cell function and on interactions between PTE and adjacent cell types (capillary endothelia and interstitial fibroblasts).

In vivo, the damage to PTE caused by a brief ischemic episode is relatively small compared to the injury inflicted when normal blood flow is resumed [49]. Some parallels can be drawn in the current *in vitro* model where hypoxia-induced changes in matrix synthesis and turnover were maintained or exacerbated on return to normoxia. Relatively short periods of post-hypoxia were studied and it is not clear whether enzyme, inhibitor and ECM protein levels would, with time, return to control or if the changes induced represent long-term alterations, possibly regulated by an autocrine mechanism(s), and leading to a chronic accumulation of ECM material. Net collagen production did continue to increase up to 72 hours post-hypoxia, suggesting long-term effects of a single hypoxic incident. While a 24 hour period of 1% oxygen is unlikely to have a direct *in vivo* parallel these studies demonstrate the potential for hypoxia-induced changes *in vivo*. Further investigations will examine the effects of longer periods of less severe hypoxia, in an attempt to mimic sustained chronic low grade hypoxia *in vivo* and determine the effects of repeated hypoxic insults to establish whether a single hypoxic episode renders the cell more susceptible to a second hypoxic 'hit' resulting in sustained injury.

It remains to be established whether the effects of hypoxia on PTE ECM metabolism are mediated by direct effects on gene expression via O_2-responsive enhancer elements (ORE) present in the gene promoters [14] or are mediated by secondary factors (such as growth factors or cytokines) induced by hypoxia [14]. Hypoxia has been reported to stimulate the production of a variety of growth factors including the pro-fibrotic factor, TGF-β, in a variety of cell types [50]. Three of the five isoforms of TGF-β (TGF-$\beta_{1\text{-}3}$) have been identified in mammalian cells [51] and TGF-β_1 has been implicated as a major fibrogenic agent in the lung, liver and kidney [51–55]. PTE produce TGF-β (mainly TGF-β_1 with smaller amounts of TGF-β_2 [56]) under normoxic conditions, suggesting this growth factor as a candidate mediator for the hypoxia-induced changes in ECM metabolism. Although hypoxia stimulated secretion of both active and latent TGF-β by PTE, the addition of the neutralizing anti-TGF-β_1 antibody did not block the hypoxia-induced decreases in gelatinolytic activity and collagen IV mRNA levels or the increases in expression of TIMP and collagen I mRNAs. Furthermore, exogenous TGF-β_1 (at concentrations equivalent to active (2.6 ng/ml) and total

TGF-β (10 ng/ml) produced by PTE exposed to hypoxia) did not mimic the spectrum of effects elicted by hypoxia (Table 1). Taken together, these data suggest that, in contrast to findings in mesangial cells in which TGF-β_1 mediates the hypoxia-induced increase in collagen IV mRNA levels [25], TGF-β_1 is not the primary mediator of the hypoxia-induced changes in PTE ECM metabolism. However, we cannot currently exclude the possibility that the changes observed in PTE ECM metabolism with hypoxia are due to TGF-β_2, the other TGF-β isoform expressed by PTE [56], since the measurement of secreted TGF-β includes all isoforms, while the neutralizing antibody used blocks only the β_1 isoform.

These studies clearly do not exclude a role for other hypoxia-induced growth factors as mediators of the effects of decreased oxygen tension on PTE ECM metabolism. PDGF may be an important candidate since it is known that the PDGF gene can be induced during oxygen-deprivation in other cell types [14] and it has been shown that tubular epithelial cells can produce this factor [3]. Another candidate is endothelin-1 (ET-1). Previous studies from our group showed that hypoxia stimulates ET-1 production by PTE, which has both autocrine and paracrine effects [19]. The VEGF gene also has an ORE [14] and increased expression of VEGF has been reported in the PTE of human kidneys in which narrowing of the glomerular vessels would lead to reduced renal oxygenation [57]. In addition, it may be that the genes of matrix proteins, such as the collagens, demonstrate oxygen sensitivity, responding directly to changes in oxygen levels via ORE [14].

In summary, this study demonstrates that hypoxia simultaneously stimulates PTE collagen production and decreases matrix turnover via decreased gelatinase activity and increased expression of TIMPs. The mechanism(s) by which these changes occur remain to be worked out but appear to be independent of TGF-β_1. Hypoxia has differential effects on collagen gene expression, suppressing collagen IV and stimulating collagen I. Thus, the increase in collagen production is due to increased expression of collagen I, an isoform more commonly associated with interstitial matrix rather than the TBM. 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 in this study support the hypothesis that hypoxic injury to PTE may be a pro-fibrogenic stimulus and may provide a common pathway by which diseases of diverse etiology produce a similar pathology. The concept of hypoxia as a modulator of matrix metabolism is clearly of importance in the kidney, but is also likely to be relevant in other organs that undergo fibrosis such as the lung, liver and skin, where ischemic injury to epithelial cells precedes the development of fibrosis.

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