

Renal expression of transforming growth factor- β inducible gene-h3 (β ig-h3) in normal and diabetic rats¹

RICHARD E. GILBERT, JENNIFER L. WILKINSON-BERKA, DAVID W. JOHNSON, ALISON COX, TINA SOULIS, LEONARD L. WU, DARREN J. KELLY, GEORGE JERUMS, CAROL A. POLLOCK, and MARK E. COOPER

University of Melbourne Department of Medicine, Austin and Repatriation Medical Centre Heidelberg, Victoria; and University of Sydney Department of Medicine, Royal North Shore Hospital, St. Leonards, New South Wales, Australia

Renal expression of transforming growth factor- β inducible gene-h3 (β ig-h3) in normal and diabetic rats.

Background. Transforming growth factor- β (TGF- β) has been implicated in the pathogenesis of a number of kidney diseases characterized by glomerulosclerosis and tubulointerstitial fibrosis. TGF- β is secreted in a latent form requiring extracellular modification to become biologically active. TGF- β inducible gene-h3 (β ig-h3) is a recently identified TGF- β -induced gene product. The present study sought to examine β ig-h3 expression in normal and diabetic rats.

Methods. β ig-h3, TGF- β 1 and α 1 (IV) collagen gene expression were assessed by Northern blot analysis and *in situ* hybridization in 20 Sprague Dawley rats, randomly assigned to receive streptozotocin (diabetic, $N = 11$) or citrate buffer alone (control, $N = 9$) and sacrificed eight months later. The effect of exogenous TGF- β 1 on β ig-h3 expression was also assessed in cultured proximal tubular cells.

Results. *In situ* hybridization localized β ig-h3 gene expression to the juxtaglomerular apparatus and the *pars recta* (S3 segment) of proximal tubules in both control and diabetic animals. Kidney TGF- β 1, β ig-h3 and α 1 (IV) collagen mRNA from diabetic rats were increased two- to threefold compared with controls ($P < 0.01$). There was a significant correlation between TGF- β 1 and β ig-h3 gene expression in kidneys from diabetic rats ($r = 0.73$, $P = 0.01$). In addition, β ig-h3 mRNA increased in response to exogenous TGF- β 1 in a dose-dependent fashion in cultured proximal tubular cells.

Conclusion. These findings support the hypothesis that biologically active TGF- β plays a pathogenetic role in diabetic kidney disease and suggest that β ig-h3 may be a useful index of TGF- β 1 bioactivity in the kidney.

Transforming growth factor- β (TGF- β) has been implicated in the pathogenesis of a number of kidney diseases

¹ See Editorial by Border and Noble, p. 1390.

Key words: transforming growth factor- β -inducible gene-h3, TGF- β , diabetes, juxtaglomerular apparatus, proximal tubule, *pars recta*.

Received for publication August 18, 1997

and in revised form April 23, 1998

Accepted for publication April 30, 1998

© 1998 by the International Society of Nephrology

characterized by glomerulosclerosis and tubulointerstitial fibrosis [1]. Of the three mammalian forms of TGF- β , TGF- β 1 has been the most widely implicated in tissue fibrosis [2]. TGF- β 1 is synthesized as a 391 amino acid precursor molecule with little biological activity. In tissues TGF- β 1 is found as a homodimer that may be associated with a TGF- β binding protein. This latent TGF- β 1 complex is present in extracellular matrix [3] where it may be activated by cleavage of its N-terminal latency associated peptide (LAP) to yield mature dimeric TGF- β 1 [4]. The mechanisms by which this activation occurs are uncertain but may include both enzymatic [5] and non-enzymatic mechanisms [6]. In addition to the complexities of TGF- β activation its biological effects may also be modified by the presence of the proteoglycan decorin [7] and the scavenging protein α 2-macroglobulin [8]. Thus, increased TGF- β 1 mRNA or protein may not necessarily reflect parallel changes in TGF- β 1 biological activity leading to the use of TGF- β 1 bio-assays in some experimental studies. These bio-assays include the measurement of TGF- β 's growth inhibitory effects on mink lung epithelial cells [9] or its ability to influence the splicing pattern of fibronectin transcripts [10]. More recently, the activity of TGF- β 1 has been assessed in human [11] and rat vessels [12] by the measurement of β ig-h3 (transforming growth factor- β -inducible gene h3), a novel TGF- β induced gene product identified by differential hybridization [13].

The present study sought to determine whether β ig-h3 is expressed in the rat kidney and to examine its relationship with TGF- β expression in controls animals and in rats with experimental diabetes. Diabetes was chosen as a model in which to study the relationship between TGF- β and β ig-h3 since several *in vitro*, animal and human studies suggest that glucose induces expression of biologically active TGF- β [14]. Furthermore, because recent studies have indicated that the proximal tubule may be an important site of TGF- β and extracellular matrix protein expression in experimental diabetes [15, 16], this study also sought to

determine the effect of exogenous TGF- β on β ig-h3 transcription in cultured proximal tubular cells.

METHODS

Animals

Twenty male Sprague Dawley rats, aged eight weeks and weighing between 200 and 250 grams, were randomly assigned to receive streptozotocin (STZ, $N = 11$) at a dose of 45 mg/kg (diabetic) or citrate buffer alone (control, $N = 9$). All rats were given free access to standard chow containing 20% protein (Clark, King & Co, Melbourne, Australia). Only STZ-treated animals with plasma glucose levels > 15 mmol/liter were considered diabetic and included in the study. Animals were sacrificed at eight months. Diabetic animals were treated with 6 units insulin zinc suspension (Ultratard HM; Novo Nordisk, Bagsvaerd, Denmark) injected subcutaneously three times per week to maintain body weight and improve long-term survival. For 24 hours prior to sacrifice, rats were housed in metabolic cages for subsequent measurement of urinary albumin excretion using a coated tube radioimmunoassay [17]. Immediately prior to sacrifice, rats were weighed. Animals were sacrificed by decapitation and blood collected for determination of plasma glucose by glucose oxidase technique [18] and hemoglobin A₁ by high performance liquid chromatography (HPLC) [19]. The left kidney was removed and bisected sagittally. The anterior half-kidney was immersion fixed in 10% neutral buffered formalin for *in situ* hybridization and the posterior half was immersed in Methyl Carnoy's fixative for immunohistochemical studies. The right kidney was snap frozen in liquid nitrogen and subsequently stored at -80°C for later Northern analysis.

Patients

For *in vitro* studies human proximal tubular cells (PTC) were used. Segments of macroscopically and histologically normal renal cortex were obtained aseptically from three adult human kidneys removed surgically because of small (< 6 cm) renal adenocarcinomas. The ages (sex) of the patients were 21 (male), 44 (male) and 72 (female). Patients were otherwise healthy and were receiving no medications. Informed consent was obtained prior to each operative procedure, and the use of human renal tissue for primary culture was approved by the Royal North Shore Hospital and University of Sydney Human Medical Research Ethics Committee.

Cell culture

Primary cultures of human proximal tubular cells (PTC) were obtained using previously described methods [20]. Briefly, renal cortical tissue was dissected from the medulla, minced, digested with collagenase (class 2, 383 U/mg; Worthington, Freehold, NJ, USA) and passed through a 100 μm mesh. Filtered tissue was resuspended in 45%

Percoll (Pharmacia, Uppsala, Sweden) and separated into four distinct bands by isopycnic ultracentrifugation. The lowermost band was removed for PTC culture and resuspended in serum-free, antibiotic-free, hormonally-defined media, consisting of 1:1 (vol/vol) Dulbecco's modified Eagle's media and Ham's F-12 (DMEM/F-12; ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), supplemented with 5 $\mu\text{g/ml}$ human transferrin (Sigma, St. Louis, MO, USA), 5 $\mu\text{g/ml}$ (0.87 μM) bovine insulin (Sigma), 0.05 μM hydrocortisone (Sigma), 10 ng/ml (1.64 nM) epidermal growth factor (Collaborative Research Inc., Bedford, MA, USA), 50 μM prostaglandin E1 (Sigma) and 5 pM triiodothyronine (Sigma). The tubular fragments were plated at a density of 1.5 mg pellet/cm² (approximately 5000 to 7000 fragments/cm²) in 75 cm² flasks (Corning, New York). Media were changed every 48 hours. The cells were incubated in humidified 95% air/5% CO₂ at 37°C and were subcultured at near-confluence using seeding densities of 4000 cells/cm². Such cells were designated passage 1. Cytologic examination of PTC preparations from all donors failed to reveal any evidence of cellular atypia. The ultrastructural, growth and transport characteristics of these cells and their responses to hormonal and cytokine stimulation have been previously studied and found to reproducibly exhibit the features of PTC *in vivo* [20].

Experimental protocol

Northern blot analyses were performed on confluent, quiescent, passage 2 human PTC grown in 6 cm diameter Petri dishes (Nunc, Roskilde, Denmark). Quiescence was achieved by incubation for 24 hours in basic media (DMEM/Ham's F-12 containing 5 $\mu\text{g/ml}$ human transferrin). Cells were then incubated for 24 hours with basic media containing either vehicle (control) or various concentrations (0.1, 1.0 and 10 ng/ml) of TGF- β 1 (Sigma). The concentrations of TGF- β 1 employed were based on previous dose-response studies in these cells [21]. To determine the specificity of action of TGF- β 1 on β ig-h3 expression, additional experiments were performed in which confluent, quiescent PTC were incubated for 24 hours with 100 ng/ml epidermal growth factor (EGF), 100 ng/ml insulin-like growth factor-I (IGF-1) or appropriate vehicles (controls). These cytokines were selected as they have both been shown, at the concentration used, to be potent stimulators of proximal tubular cell growth [22, 23] and matrix expression [24, 25] and have also been shown to be increased in the kidney in experimental diabetes [26, 27].

Northern analysis

Kidneys stored at -80°C were homogenized (Ultra-Turrax; Janke and Kunkel, Staufen, Germany) and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [28]. RNA purity and concentration were determined spectrophotometrically. Twenty microgram samples were denatured and

electrophoresed through 0.8% agarose formaldehyde gels. RNA integrity was verified by examination of the 28S and 18S ribosomal RNA bands of ethidium bromide stained material under ultraviolet light. RNA was then transferred onto nylon filters (Hybond-N, Amersham, UK) by capillary action and fixed by ultraviolet irradiation.

Filters were hybridized with a 2055 bp cDNA probe coding for human β ig-h3 (gift of Dr. K. Bennett, Bristol-Myers Squibb, Seattle, WA, USA), a 985 bp cDNA probe coding for rat TGF- β 1 (gift of Dr. Qian, NIH, Bethesda, MD, USA) and a 1.8 kb cDNA probe coding for mouse α 1 (IV) collagen [α (1) IV col; gift of Dr. R. Timpl, Max Plank, Martinsried, Germany]. cDNA probes were labeled with [α - 32 P] dCTP (DuPont, Boston, MA, USA) by random primed DNA synthesis (Boehringer Mannheim, Mannheim, Germany). Hybridization of filters was performed at 42°C for 24 hours in 50% formamide, 45 mM Na₂HPO₄, 5 × Denhardt's solution, 0.5% SDS and sonicated salmon sperm DNA. Filters were then washed in solutions of decreasing ionic strength and increasing temperature. The final stringency was 0.1 × standard saline citrate (SSC) with 0.1% SDS for 20 minutes at 42°C. Intensity of hybridization was quantified by scanning densitometry (LKB Ultrosan XL, Bromma, Sweden). To control for differences in RNA loading and transfer filters were hybridized with an oligonucleotide probe for 18S rRNA end-labeled with [α - 32 P] dCTP (DuPont) by terminal transferase (Boehringer Mannheim). Results were expressed as the ratio of image intensity of mRNA to 18S rRNA relative to control kidneys which were arbitrarily assigned a value of 1.

In situ hybridization

The cDNA probe for β ig-h3 was cloned into pBluescript KS+ (Stratagene) and linearized with *Hind*III and *Xba*I to produce an antisense riboprobe with T3 RNA polymerase. *In situ* hybridization was performed as previously described [29]. In brief, 4 μ m thick sections cut from formalin-fixed paraffin-embedded kidney tissue were placed onto slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in P buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and incubated in 125 μ g/ml Pronase E in P buffer for 10 minutes at 37°C. Sections were then washed in 0.1 M sodium phosphate buffer (pH 7.2), briefly refixed in 4% paraformaldehyde for 10 minutes, rinsed in milliQ water, dehydrated in 70% ethanol and air dried. Hybridization buffer containing 2 × 10⁴ cpm/ μ l riboprobe in 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM Na₂HPO₄, 5 mM EDTA (pH 8.0), 1 × Denhardt's solution, 50% formamide, 17 mg/ml yeast RNA, 10% wt/vol dextran sulfate was heated to 85°C for five minutes. Twenty-five microliters of this solution were then added to each section. Hybridization was performed overnight at 60°C in 50% formamide humidified chambers. Sections hybridized with sense probe

for β ig-h3 were used as controls for non-specific binding. After hybridization, slides were washed in 2 × SSC containing 50% formamide prewarmed to 50°C to remove coverslips. Sections were then washed in the above solution for one hour at 55°C, rinsed three more times in RNase buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.5 M NaCl) and then incubated with RNase A (150 μ g/ml) for one hour at 37°C. Sections were later washed in 2 × SSC for 45 minutes at 55°C, dehydrated in graded ethanol, air dried and exposed to Kodak X-Omat autoradiographic film for one to three days. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box with desiccant at room temperature for two to three weeks, immersed in Kodak D19 developer, fixed in Ilford Hypam and stained with hematoxylin and eosin or periodic acid-Schiff (PAS).

Immunohistochemistry

Four-micrometer thick sections were placed onto slides, deparaffinized and rehydrated. Sections for type IV collagen immunohistochemistry underwent microwave oven pretreatment as previously described [30]. To block endogenous peroxidase sections were pretreated with 1% H₂O₂/methanol. Sections were next incubated in Protein Blocking Agent (Lipshaw-Immuron, Pittsburgh, PA, USA) for 20 minutes at room temperature. This was followed by incubation with polyclonal goat anti-bovine/anti-human type IV collagen antibody (Southern Biotechnology, Birmingham, AL, USA) for 60 minutes at room temperature, washing sections in PBS and incubating them with universal biotinylated immunoglobulin (DAKO, Carpinteria, CA, USA) and peroxidase-conjugated streptavidin (DAKO) as previously described [12]. Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Sections were then counterstained with Mayer's hematoxylin and examined by two independent observers blinded to the disease status of the animal. Immunostaining was scored using a scale of 0 to 3 where 0 = no staining and 3 = maximum staining (adapted from [15]). Negative controls included omitting the primary antibody or replacing it with normal rabbit IgG at an equivalent protein concentration.

Statistics

Because of a positively skewed distribution albuminuria was logarithmically transformed before statistical analysis. Results are expressed as mean \pm SEM unless stated otherwise. Data derived from immunohistochemical studies were not normally distributed and were analyzed non-parametrically using the Mann-Whitney *U*-test. All other comparisons between control and diabetic groups were analyzed by ANOVA with correction for multiple comparisons using the Fisher's least significant difference test. Correlation was examined by linear regression analysis. Analyses were performed using the Statview SE+ Graphics package

Table 1. Animal characteristics at end of study period

	Control	Diabetic
N	9	11
Body weight g	575 \pm 25	428 \pm 23 ^b
Blood pressure mm Hg	118 \pm 2	140 \pm 3 ^b
Glucose mmol/liter	4.3 \pm 0.1	24.1 \pm 1.6 ^b
HbA _{1c} %	1.8 \pm 0.2	4.8 \pm 0.2 ^b
Albuminuria μ g/day	2844 \pm 554	82742 \pm 16821 ^b
Type IV collagen ^c	1 (1)	2.5 (1.5–3.0) ^a

Data are means \pm SEM or median (range).

^a $P = 0.05$ versus control

^b $P < 0.001$ versus control

^c Semiquantitative scoring for immunohistochemistry

(Abacus Concepts, Calabasas, CA, USA) on an Apple Macintosh Quadra 605 (Apple Computer Inc, Cupertino, CA, USA). A P value ≤ 0.05 was considered statistically significant.

RESULTS

Animal characteristics

Plasma glucose and HbA_{1c} confirmed the presence of diabetes in all STZ-treated rats (Table 1). Diabetes was associated with reduced body mass and increased blood pressure when compared with control animals (Table 1). The albumin excretion rates were at least tenfold higher in diabetic compared with non-diabetic animals (Table 1).

Northern analysis: *In vivo* studies

Kidney TGF- β 1 and β ig-h3 mRNA from diabetic rats were both increased approximately twofold compared with their non-diabetic counterparts (Figs. 1 and 2). A significant correlation between TGF- β 1 and β ig-h3 gene expression was present in kidneys from diabetic ($r = 0.73$, $P = 0.01$; Fig. 3) and approached statistical significance in control rats ($P = 0.08$). Northern analysis also demonstrated that type IV collagen mRNA was increased in the diabetic rat kidney. A close correlation between β ig-h3 and α (1) IV collagen mRNA in both control ($r = 0.92$, $P < 0.05$) and diabetic rat kidneys ($r = 0.79$, $P < 0.05$) was also noted.

Northern analysis: *In vitro* studies

Under basal conditions only low levels of β ig-h3 transcript were detected. However, β ig-h3 mRNA increased in response to exogenous TGF- β 1 in a dose-dependent fashion (Figs. 4 and 5). In contrast, β ig-h3 mRNA was unchanged in response to either 100 ng/ml EGF or IGF-I (Fig. 6).

In situ hybridization

In situ hybridization demonstrated β ig-h3 gene expression in the outer stripe of the outer medulla (OSOM) and inner cortex in kidneys of both control and diabetic animals (Fig. 7). Light microscopic examination of emulsion-dipped

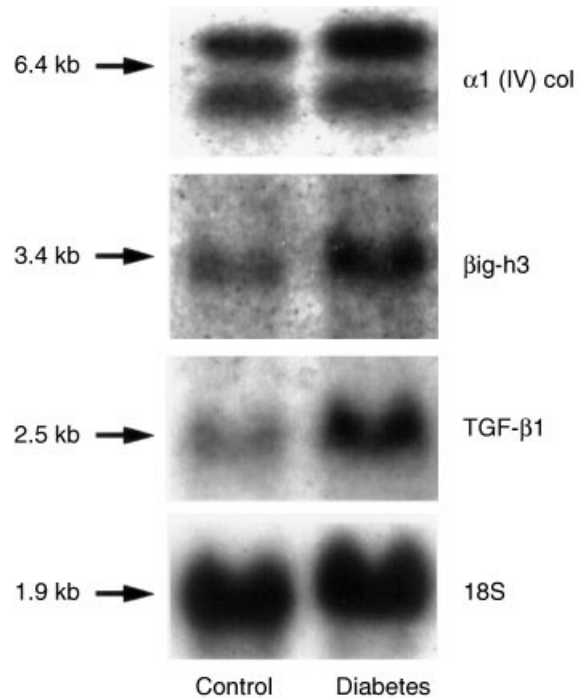


Fig. 1. Northern blot of β ig-h3, TGF- β 1, α 1 (IV) collagen and 18S in control and diabetic rat kidneys. Increased gene expression of β ig-h3 and TGF- β 1 is seen in kidneys of diabetic rats. 18S rRNA is similar in control and diabetic groups.

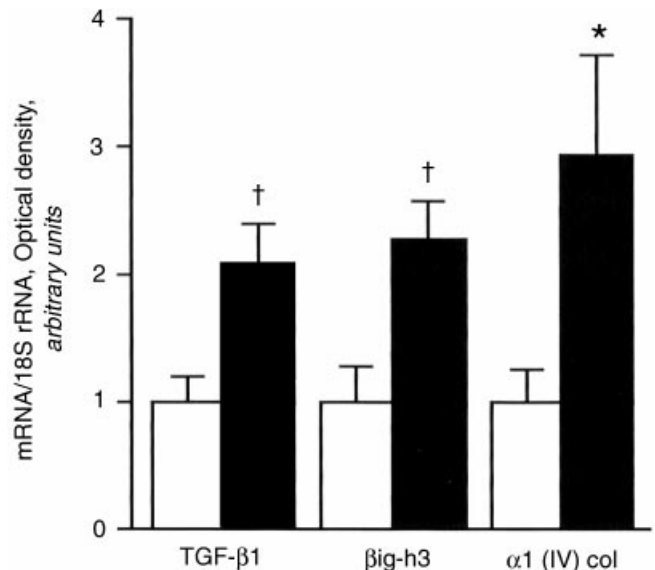


Fig. 2. Quantitation of kidney β ig-h3, TGF- β 1 and α 1 (IV) collagen mRNA in control (\square) and diabetic (\blacksquare). Data are means \pm SEM of the ratio of optical density of β ig-h3, TGF- β 1 and α 1 (IV) col mRNA to that of 18S rRNA relative to control values (designated an arbitrary value of 1). * $P = 0.05$, † $P < 0.01$, diabetic versus control

sections revealed that β ig-h3 mRNA was localized to the vascular component of the juxtaglomerular apparatus (JGA) (Fig. 8 A, B) in control and in greater abundance in diabetic rats. In the outer cortex, glomeruli, proximal and

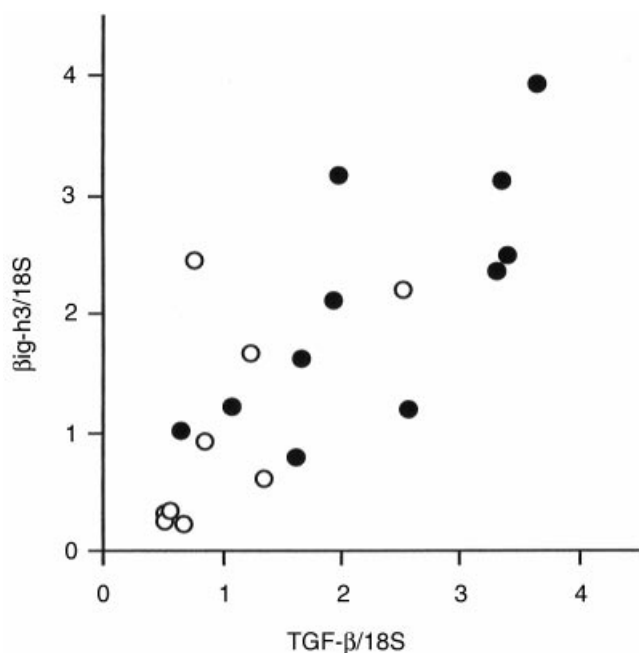


Fig. 3. Correlation between β ig-h3 and TGF- β mRNA in control (○) and diabetic (●) rat kidneys.

distal tubules, collecting ducts and maculae densa showed no specific hybridization. Within the proximal tubules intense β ig-h3 expression was only present in the inner cortex (Fig. 8 C, D) and OSOM, the location of the pars recta (S3). In the inner stripe of the outer medulla and in inner medulla no specific hybridization was detected. No difference in the sites of distribution within the kidney was noted between control and diabetic animals. No hybridization was detected with β ig-h3 sense riboprobe (Fig. 8 E, F).

Immunohistochemistry

Greater immunostaining for type IV collagen was present in the interstitium of diabetic rats compared with control animals ($P < 0.05$; Table 1 and Fig. 9). Tissues treated with normal rabbit IgG showed no positive staining.

DISCUSSION

The present study demonstrates that β ig-h3 is constitutively expressed in the vascular component of the juxtaglomerular apparatus and in the *pars recta* (S3 segment) of the proximal tubule. In addition, β ig-h3 was overexpressed in parallel with TGF- β 1 in the kidneys of rats with experimental diabetes.

β ig-h3 is a 683 amino acid secreted protein that was recently cloned from a human adenocarcinoma cell line that had been treated with TGF- β 1 and screened by differential hybridization [13]. The mature protein contains a secretory sequence in its NH₂ terminus, four homologous

internal domains and an integrin recognition sequence (arg-gly-asp) at its carboxyl end [13]. Sequence analysis of β ig-h3 demonstrates significant evolutionary conservation with 91% homology at the amino acid level between murine and human forms of the protein [31]. While the primary structure of β ig-h3 is unique, it does have regions of homology with insect fascilin-I and *Mycobacterium bovis* MPB70 [13].

The physiological functions of β ig-h3 are not well understood though preliminary investigations suggest that it may modulate tumor formation and cell adhesion [31, 32]. Indeed, recent studies suggest that β ig-h3 may be an extracellular matrix microfibril-associated protein (MP 78/80) that binds to the microfibrillar proteins fibrillin-I and MAGP-1 [33]. *In vitro*, increased β ig-h3 expression in response to TGF- β has been demonstrated in a variety of cell lines including mammary epithelial cells, fibroblasts and keratinocytes [31]. *In vivo*, β ig-h3 has been found in the papillary dermis [32], corneal epithelium [34] and blood vessels [11, 12].

Investigation of the role of TGF- β in diabetic kidney disease has largely focused on the glomerulus, although the tubulointerstitium also undergoes significant structural damage [35]. *In vitro*, exposure of proximal tubular cells to high glucose concentrations leads to increased TGF- β 1 and collagen expression [36] as it does in mesangial cells [37]. Indeed, TGF- β 1 is found throughout the nephron including the *pars recta* of the proximal tubule [38] and receptors for TGF- β are ubiquitously expressed [39]. Thus, the close proximity of ligand and receptor in the region of the proximal tubule (as at many other sites) is consistent with a paracrine action of TGF- β with a high glucose-mediated elevation in TGF- β 1 leading to increased expression of the TGF- β inducible gene β ig-h3, as demonstrated in the present study. The mechanisms underlying the restricted pattern of β ig-h3 expression are not understood, though highly localized patterns of gene transcription in the kidney are well described [40] and presumably reflect the various cell phenotypes within the nephron.

As with diabetic nephropathy in humans, experimental diabetes is associated with increased extracellular matrix deposition as indicated in the present study by the increased gene expression and tissue deposition of type IV collagen. While TGF- β stimulates the synthesis of extracellular matrix and, in general, inhibits cell proliferation, it is not the only growth factor with these actions [41]. Furthermore, the complexities of TGF- β activation following its secretion have led to various strategies of assessing its biological activity. For instance, since TGF- β 1 causes preferential expression of certain alternatively spliced isoforms of fibronectin mRNA [42], measurement of such transcripts has been used to assess TGF- β 1 biological activity [10]. However, this alternative splicing of fibronectin mRNA is not unique to TGF- β 1 but is also found in response to

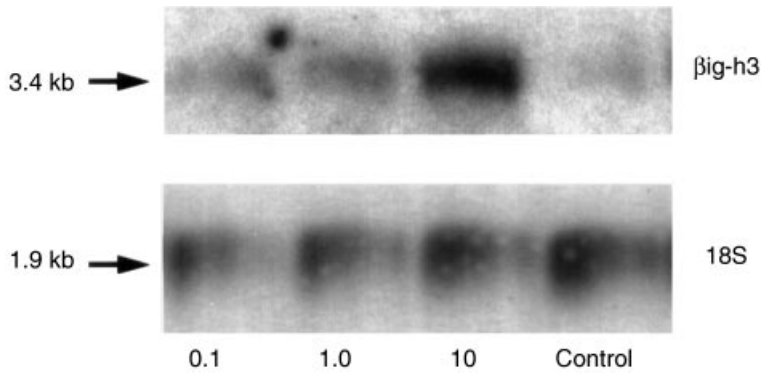


Fig. 4. Northern blot of β ig-h3 gene expression in cultured proximal tubular cells exposed to TGF- β 1 at concentrations of 0 to 10 ng/ml. β ig-h3 mRNA increased in response to exogenous TGF- β 1 in a dose-dependent fashion. 18S rRNA expression was unchanged.

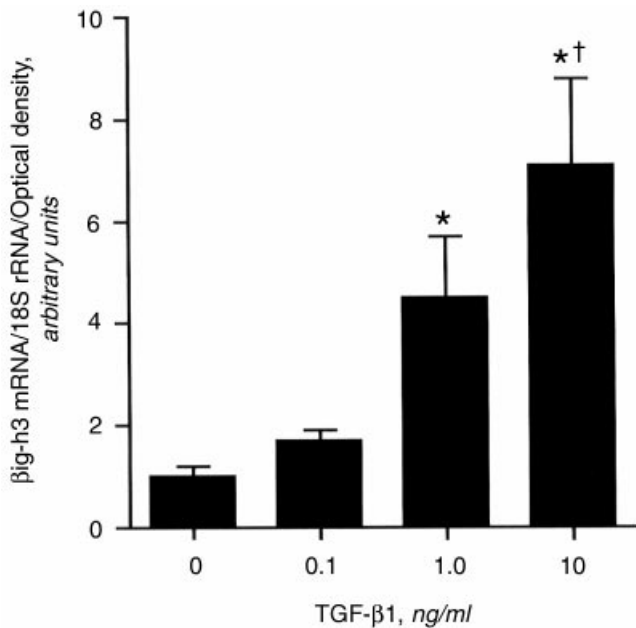


Fig. 5. β ig-h3 gene expression in cultured proximal tubular cells exposed to TGF- β 1 at concentrations of 0 to 10 ng/ml. Data are means \pm SE of the ratio of optical density of β ig-h3 to that of 18S rRNA relative to control values (designated an arbitrary value of 1). * P < 0.05 versus control, † P < 0.05 versus 0.1 ng/ml.

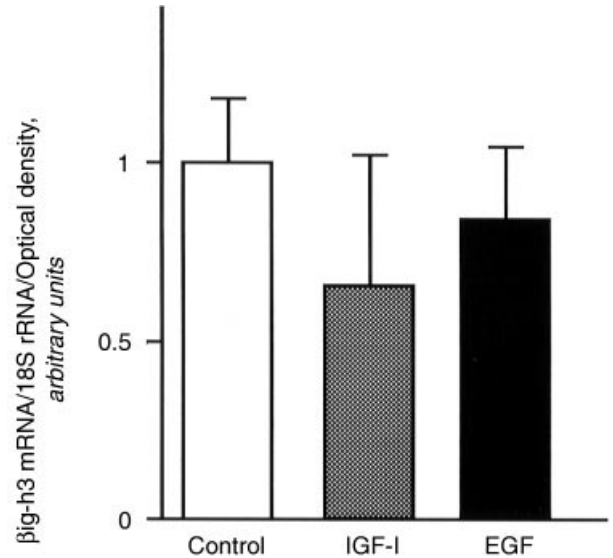


Fig. 6. β ig-h3 gene expression in cultured proximal tubular cells exposed to EGF and IGF-I 100 ng/ml. Data are means \pm SE of the ratio of optical density of β ig-h3 to that of 18S rRNA relative to control values (designated an arbitrary value of 1).

other growth factors such as retinoic acid and 1,25-dihydroxy vitamin D₃ [43]. The growth inhibitory action of TGF- β on mink lung epithelial cell ³H-thymidine incorporation has also been used as a TGF- β bioassay [9]. However, other growth factors such as hepatocyte growth factor (HGF) may release these cells from the growth inhibitory effects of TGF- β [44]. This may be particularly relevant to diabetes where *in vitro* and *in vivo* studies indicate increased expression of many pro-proliferative growth factors including HGF [45, 46]. In the present study diabetes was associated with increased expression of both TGF- β 1 and β ig-h3. In addition, there was a significant correlation between TGF- β 1 and β ig-h3 and between β ig-h3 and α (1) IV collagen mRNA consistent with translation of TGF- β 1 to biologically active protein. Furthermore, a dose-depen-

dent relationship between the magnitude of β ig-h3 expression and the concentration of TGF- β 1 in cultured proximal tubular cells was also noted. In contrast, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), two cytokines that are potent stimulators of proximal tubular cell growth [22] and matrix synthesis [24, 25] and have also been shown to be increased in the kidney in experimental diabetes [26, 27] did not affect β ig-h3 expression. These findings suggest that β ig-h3 may be useful as an index of TGF- β 1 bioactivity in the kidney as well as in blood vessels [11, 12]. However, while the expression of β ig-h3 is linked to TGF- β bio-activity, and its expression was unaffected by EGF or IGF-I, it remains to be established whether induction of expression of β ig-h3 is exclusively TGF- β -dependent and whether the measurement of β ig-h3 gene expression is superior to more conventional methods of assessing TGF- β bio-activity.

Within the renal cortex β ig-h3 gene expression was

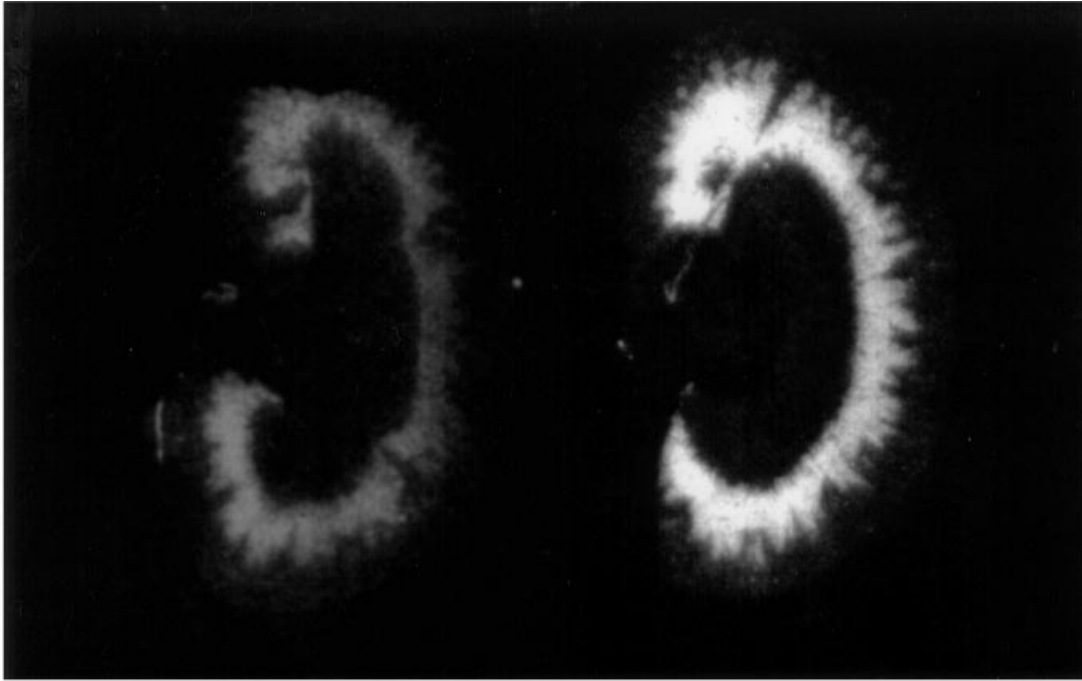


Fig. 7. *In situ* hybridization for β ig-h3 mRNA in longitudinal sections of control (left panel) and diabetic (right panel) rat kidney localizing gene expression to the inner cortex and outer stripe of outer medulla.

localized to the juxtaglomerular apparatus (JGA). This region of the nephron, which links the distal end of the thick ascending limb of the loop of Henle to the vascular pole of the glomerulus, is intimately involved with the regulation of tubuloglomerular feedback, glomerular capillary pressure and renin secretion [47]. Within the JGA, β ig-h3 mRNA was not present in the macula densa, but was found exclusively in cells of the vascular component. These highly specialized cells are the source of various vasoactive factors including renin [48], angiotensin II [49] and nitric oxide synthase [50]. In addition, expression of both TGF- β 1 [51] and platelet-derived growth factor (PDGF) [52] have been demonstrated in the vascular cells of the JGA in cyclosporine-induced nephrotoxic injury, and increased TGF- β 2 has also been found in the JGA in response to volume [53] and potassium depletion [54]. Interaction between vasoactive factors and TGF- β has also been suggested by the ability of TGF- β to stimulate renin release [55] and the action of angiotensin II in increasing TGF- β expression [56]. Indeed, experimental diabetes is associated with both increased expression of renin [57] and TGF- β 1 [10, 16] in the kidney.

β ig-h3 was also expressed in the inner cortex and outer stripe of the outer medulla where it was found exclusively in proximal tubular cells as identified morphologically by their cuboidal shape, eosinophilic cytoplasm and brush border. These proximal tubules at the outer stripe of the outer medulla are almost exclusively the S3 segment or *pars recta* [58]. The physiological significance of the restricted expres-

sion of β ig-h3 to this part of the nephron is uncertain. The *pars recta* of the proximal tubule may be particularly vulnerable to nephrotoxic and ischemic injury as a consequence of tubular concentration, interstitial hypertonicity and low oxygen tension [59]. This has led to the suggestion that ischemic peritubular microangiopathy in diabetes may preferentially affect the *pars recta* of the proximal tubule [60]. In a study of extracellular matrix gene expression in STZ-diabetes, Ihm and colleagues reported increased gene expression of α 1 (IV) collagen predominantly in the proximal tubules of the deep cortex and outer medullary stripe at 28 but not seven days of diabetes, suggesting that the described collagen overexpression was a diabetes rather than a STZ effect [61]. In a recent study of patients with incipient diabetic nephropathy, Nuyts and coworkers reported a significant correlation between glycated hemoglobin and urinary excretion of the intestinal alkaline phosphatase (hIAP), the isoenzyme expressed exclusively by the *pars recta* of the proximal tubule [60]. These findings suggest that increased expression of both hIAP and β ig-h3 may reflect the response of the *pars recta* to the metabolic insult of hyperglycemia. In addition, this segment of the proximal tubule is also the major intrarenal location of neutral endopeptidase [62], the enzyme responsible for the metabolism of atrial natriuretic peptide [63], bradykinin [64] and endothelin [65]. Indeed, a link between the intrarenal actions of vasoactive hormones and that of TGF- β is also suggested by the finding that like β ig-h3, renin is constitutively produced only in the juxtaglomerular

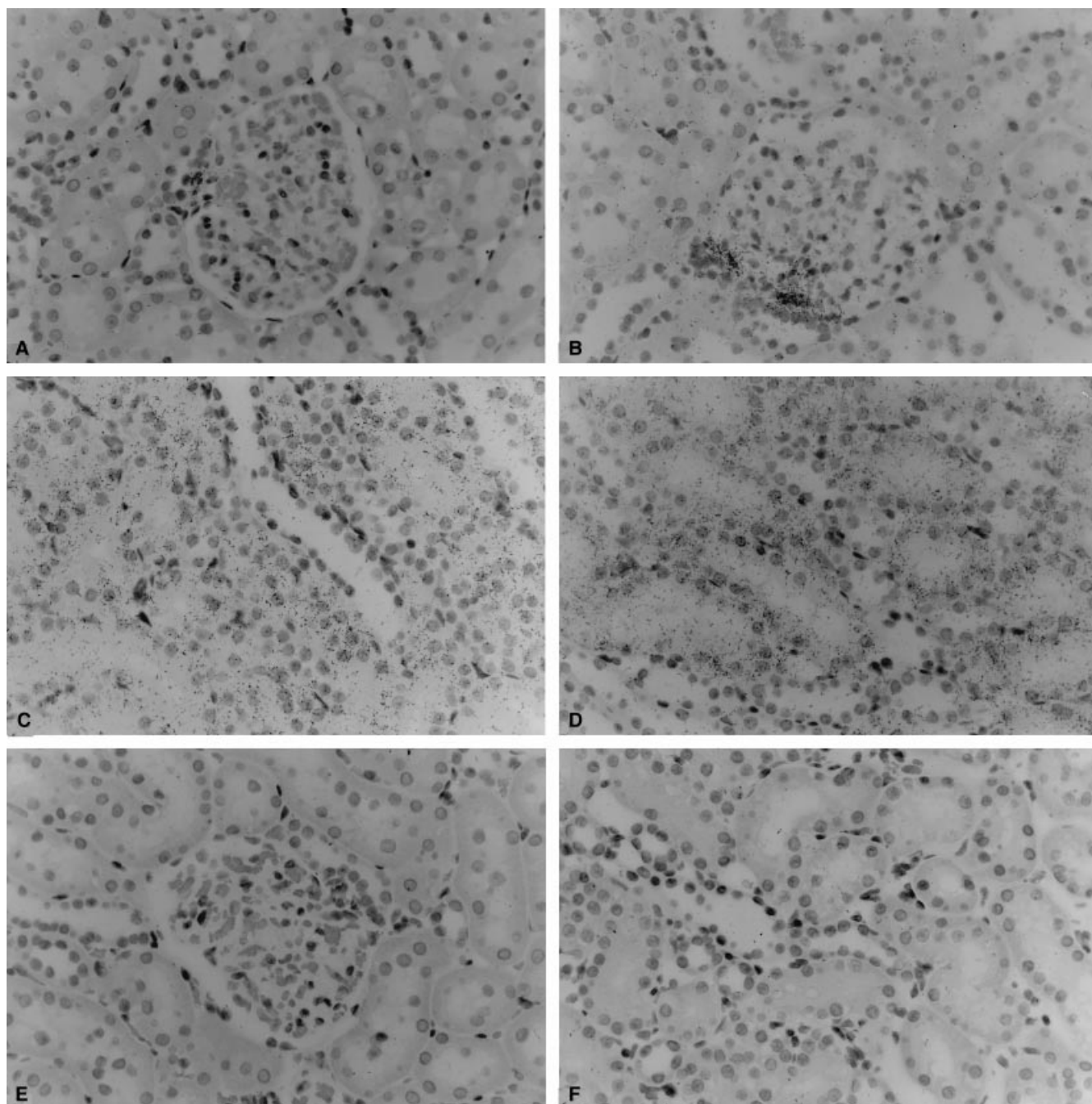


Fig. 8. *In situ* hybridization photomicrographs of β ig-h3 mRNA. In the cortex hybridization is present in the vascular component of the juxtaglomerular apparatus in control (A) and in greater abundance in diabetic (B) rat kidneys. Glomeruli, tubules and macula densa showed no β ig-h3 expression. In both control (C) and diabetic (D) kidneys proximal tubules in the inner cortex but not the outer cortex (A, B) intensely expressed β ig-h3. No specific hybridization was detected in sections exposed to β ig-h3 sense probe (E, F). Magnification $\times 400$.

apparatus and the proximal tubule [66], and, in particular, the S3 segment [67]. This interaction between the renin-angiotensin system and the effects of TGF- β overexpression may be particularly relevant to diabetic nephropathy where experimental studies and clinical trials indicate a beneficial effect of angiotensin converting enzyme inhibition in reducing TGF- β [16, 68] in addition to ameliorating

the associated structural and functional abnormalities of diabetic renal disease [16, 69].

In summary, the present study supports the contention that biologically active TGF- β plays a pathogenetic role in diabetic kidney disease and suggests that β ig-h3 may be useful as an index of TGF- β 1 bioactivity in the kidney. However, the function of β ig-h3 in renal physiology and its

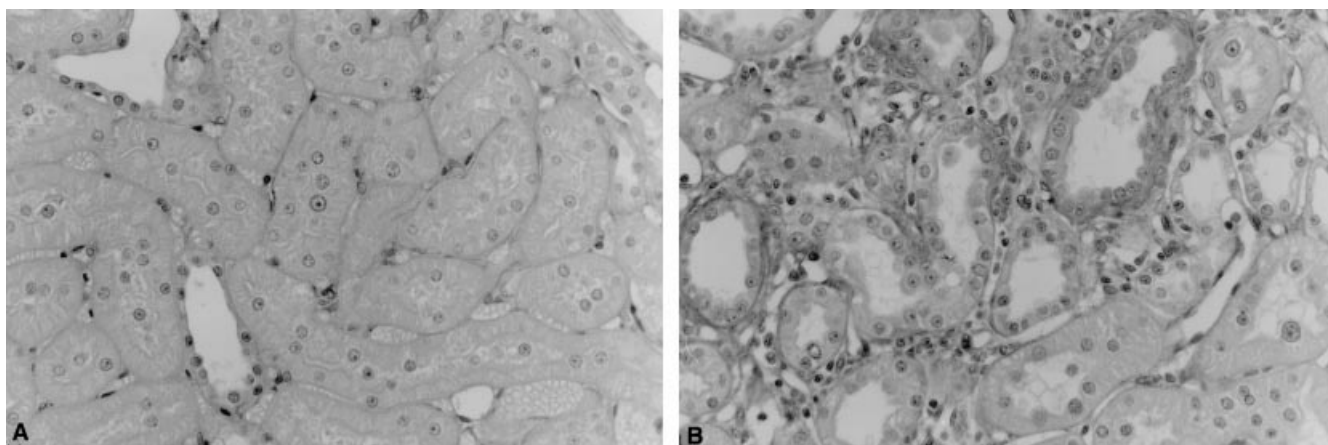


Fig. 9. Immunohistochemistry of type IV collagen in tubulointerstitium of control (A) and diabetic rats (B). Increased immunostaining is present in kidneys from diabetic rats compared with control animals. Magnification $\times 400$.

actions in various pathological states such as diabetes remain speculative. Its sites of distribution within the kidney and its association with TGF- β 1 expression raise the possibility of an interaction between vasoactive factors, matrix synthesis and the diabetic state.

ACKNOWLEDGMENTS

This work was supported by a grant from the Juvenile Diabetes Foundation International. Dr. Gilbert is the recipient of a Career Development Award from the Juvenile Diabetes Foundation International.

Reprint requests to Dr. Richard Gilbert, MB, BS, Ph.D., Department of Medicine, Austin and Repatriation Medical Centre, Repatriation Campus, Building 24, Waterdale Road, West Heidelberg 3081, Australia.
E-mail: gilbert@austin.unimelb.edu.au

APPENDIX

Abbreviations used in this article are: α 1-col, α 1-collagen; β ig-h3, transforming growth factor- β inducible gene-h3; DAB, diaminobenzidine tetrahydrochloride; EGF, epidermal growth factor; HGF, hepatocyte growth factor; HPLC, high performance liquid chromatography; IGF-1, insulin-like growth factor-1; JGA, juxtaglomerular apparatus; OSOM, outer stripe of the outer medulla; PTC, proximal tubular cells; STZ, streptozotocin; TGF, transforming growth factor.

REFERENCES

- SHARMA K, ZIYADEH FN: The emerging role of transforming growth factor- β in kidney diseases. *Am J Physiol* 266:F829-F842, 1994
- BORDER WA, NOBLE NA: Transforming growth factor- β in tissue fibrosis. *N Engl J Med* 331:1286-1392, 1994
- TAIPALE J, SAARINEN J, HEDMAN K, KESKI-OJA J: Latent transforming growth factor- β 1 and its binding protein are components of the extracellular matrix microfibrils. *J Histochem Cytochem* 44:875-889, 1996
- SHARMA K, ZIYADEH FN: The transforming growth factor- β system and the kidney. *Semin Nephrol* 13:116-128, 1993
- SCHULTZ-CHERRY S, CHEN H, MOSHER DF, MISENHEIMER TM, KRUTZSCH HC, ROBERTS DD, MURPHY-ULRICH JE: Regulation of transforming growth factor- β activation by discrete sequences of thrombospondin 1. *J Biol Chem* 270:7304-7310, 1995
- OREFFO RO, MUNDY GR, SAYEDIN SM: Activation of latent bone-derived latent TGF- β complex by isolated osteoclasts. *Biochem Biophys Res Commun* 158:817-823, 1989
- YAMAGUCHI Y, MANN DM, RUOSLAHTI E: Negative regulation of transforming growth factor- β by the proteoglycan decorin. *Nature* 346:281-284, 1990
- O'CONNOR-McCOURT MD, WAKEFIELD LM: Latent transforming growth factor β in serum. *J Biol Chem* 262:14090-14099, 1987
- GIBBONS GH, PRATT RE, DZAU VJ: Vascular smooth muscle hypertrophy vs hyperplasia. *J Clin Invest* 90:456-461, 1992
- YAMAMOTO T, NAKAMURA T, NOBLE NA, RUOSLAHTI E, BORDER WA: Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci USA* 90:1814-1818, 1993
- O'BRIEN ER, BENNETT KL, GARVIN MR, ZDERIC TW, HINOHARA T, SIMPSON JB, KIMURA T, NOBUYOSHI M, MIZGALA H, PURCHIO A, SCHWARTZ SN: β ig-h3, transforming growth factor-beta-inducible gene, is overexpressed in atherosclerotic and restenotic human vascular lesions. *Arterioscler Thromb Vasc Biol* 16:576-584, 1996
- RUMBLE JR, COOPER ME, SOULIS T, COX A, WU L, YOUSSEF S, JASIK M, JERUMS G, GILBERT RE: Vascular hypertrophy in experimental diabetes: Role of advanced glycation end products. *J Clin Invest* 99:1016-1027, 1997
- SKONIER J, NEUBAUER M, MADISEN L, BENNETT K, PLOWMAN G, PURCHIO AF: cDNA cloning and sequence analysis of β ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor- β . *DNA Cell Biol* 11:511-522, 1992
- SHARMA K, ZIYADEH FN: Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. *Diabetes* 44:1139-1146, 1995
- PARK I-S, KIYOMOTO H, ABOUD SL, ABOUD HE: Expression of transforming growth factor- β and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 46:473-480, 1997
- GILBERT RE, COX A, WU LL, ALLEN TJ, HULTHEN L, JERUMS G, COOPER ME: Expression of transforming growth factor- β 1 and type IV collagen in the renal tubulointerstitium in experimental diabetes: Effects of angiotensin converting enzyme inhibition. *Diabetes* 47:414-422, 1998
- JERUMS G, ALLEN TJ, COOPER ME: Triphasic changes in selectivity with increasing proteinuria in type I and type II diabetes. *Diabetic Med* 6:772-779, 1989
- SCHMIDT FH: Enzymatic determination of glucose and fructose simultaneously. *Klin Wochenschr* 39:1244-1247, 1961
- ALLEN TJ, COOPER ME, O'BRIEN RC, BACH LA, JACKSON B, JERUMS G: Glomerular filtration rate in the streptozotocin diabetic rat: The role of exchangeable sodium, vasoactive hormones and insulin therapy. *Diabetes* 38:1182-1190, 1990
- JOHNSON DW, BREW BK, PORONNIK P, COOK DI, FIELD MJ, POLLOCK

- CA: Transport characteristics of human proximal tubule cells in primary culture. *Nephrology* 3:183-194, 1997
21. POLLOCK CA, JOHNSON DW, BREW BK, PORONNIK P, COOK DI, FIELD MJ, GYORY AZ: Effects of insulin-like growth factor-I (IGF-I) and transforming growth factor- β (TGF β) on human proximal tubule cell (PTC) growth and sodium-hydrogen exchange (NHE). (abstract) *J Am Soc Nephrol* 7:1774, 1996
 22. KANDA S, NOMATA K, SAHA PK, NISHIMURA N, YAMADA J, KANATAKE H, SAITO Y: Growth factor regulation of renal cortical tubular cells by epidermal growth factor, insulin-like growth factor I, acidic and basic fibroblast growth factors and transforming growth factor- β in serum free culture. (abstract) *Cell Biol Int Rep* 13:687, 1989
 23. NOBES M, POLLOCK C, HENG P, FIELD M: Modulators of growth in primary culture of rat proximal tubular cells. *Nephrology* 1:65-72, 1995
 24. CREELY JJ, DIMARI SJ, HOWE AM, HYDE CP, HARALSON MA: Effect of epidermal growth factor on collagen synthesis by an epithelioid cell line derived from normal rat kidney. *Am J Pathol* 136:1247-1257, 1990
 25. SCREIBER BD, HUGHES ML, GROGGER GC: Insulin-like growth factor-1 stimulates production of mesangial cell matrix components. *Clin Nephrol* 43:368-374, 1995
 26. GILBERT RE, COX A, MCNALLY PG, DZIADEK M, WU LL, COOPER ME, JERUMS G: Increased epidermal growth factor expression in diabetes related kidney growth. *Diabetologia* 40:778-785, 1997
 27. BACH LA: IGF-I and IGF binding proteins in diabetes-related kidney growth. *Growth Regul* 2:30-39, 1992
 28. CHOMCZYNSKI P, SACCHI N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159, 1987
 29. GILBERT RE, MCNALLY PG, COX A, DZIADEK M, RUMBLE J, COOPER ME, JERUMS G: SPARC gene expression is reduced in early diabetes related kidney growth. *Kidney Int* 48:1216-1225, 1995
 30. CATTORETTI G, PILERI S, PARRAVICINI C, BECKER MH, POGGI S, BIFULCO C, KEY G, D'AMATO L, SABATTINI E, FEUDALE E, REYNOLDS F, GERDES J, RILKES F: Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol* 171:83-98, 1993
 31. SKONIER J, BENNETT K, ROTHWELL V, KOSOWSKI S, PLOWMAN G, WALLACE P, EDELHOFF S, DISTECHE C, NEUBAUER M, MARQUARDT H: β ig-h3: A transforming growth factor-beta-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice. *DNA Cell Biol* 13:571-584, 1994
 32. LEBARON RG, BEZVERKOV KI, ZIMMER MP, PAVELEC R, SKONIER J, PURCHIO AF: β ig-H3, a novel secretory protein inducible by transforming growth factor- β , is present in normal skin and promotes the adhesion and spreading of dermal fibroblasts in vitro. *J Invest Dermatol* 104:844-849, 1995
 33. GIBSON MA, HATZINIKOLAS G, KUMARTILAKE JS, SANBERG LB, NICHOLL JK, SUTHERLAND GR, CLEARY EG: Further characterization of proteins associated with elastic fiber microfibrils including the molecular cloning of MAGP-2 (MP-25). *J Biol Chem* 271:1096-1103, 1996
 34. ESCRIBANO J, HERNANDO N, GHOSH S, CRABB J, COCA-PRADOS M: cDNA from human ocular ciliary epithelium homologous to β ig-h3 is preferentially expressed as an extracellular protein in the corneal epithelium. *J Cell Physiol* 160:511-521, 1994
 35. LANE PH, STEFFES MW, FIORETTO P, MAUER SM: Renal interstitial expansion in insulin-dependent diabetes mellitus. *Kidney Int* 43:661-667, 1993
 36. ROCCO MV, CHEN Y, GOLDFARB S, ZIYADEH FN: Elevated glucose stimulates TGF-beta gene expression and bioactivity in proximal tubule. *Kidney Int* 41:107-114, 1992
 37. WOLF G, SHARMA K, CHEN Y, ERICKSEN M, ZIYADEH FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF- β . *Kidney Int* 42:647-656, 1992
 38. ANDO T, OKUDA S, TAMAKI K, YOSHITOMI K, FUJISHAMA M: Localization of transforming growth factor and latent transforming growth factor- β binding protein in rat kidney. *Kidney Int* 47:733-739, 1995
 39. WAKEFIELD LM, SMITH DM, MASUI T, HARRIS CC, SPORN MB: Distribution and modulation of the cellular receptors for transforming growth factor-beta. *J Cell Biol* 105:965-975, 1987
 40. PRICE GJ, BERKA JL, EDMONDSON SR, WERTHER GA, BACH LA: Localization of mRNAs for insulin-like growth factor binding proteins 1 to 6 in rat kidney. *Kidney Int* 48:402-411, 1995
 41. KOVACS EJ, DIPIETRO LA: Fibrogenic cytokines and connective tissue production. *FASEB J* 8:854-861, 1994
 42. BORSI L, CASTELLANI P, RISSO AM, LEPRINI A, ZARDI L: Transforming growth factor- β regulates the splicing pattern of fibronectin messenger RNA precursor. *FEBS Lett* 261:175-178, 1990
 43. MAGNUSON VL, YOUNG M, SCHATTEBERG DG, MANCINI MA, CHEN D, STEFFENSEN B, KLEBE RJ: The alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors. *J Biol Chem* 266:14654-14662, 1991
 44. TAIPALE J, KESKI-OJA J: Hepatocyte growth factor releases epithelial and endothelial cells from growth factor arrest by transforming growth factor- β 1. *J Biol Chem* 271:4342-4348, 1996
 45. NAKAMURA T, FUKUI M, EBHARA I, OSADA S, NAGAOKA I, TOMINO Y, KOIDE H: mRNA expression of growth factors in glomeruli from diabetic rats. *Diabetes* 42:450-456, 1993
 46. COUPER J, LITTLEFORD KD, COUPER RT, NAKAMURA T, FERRANTE A: High glucose and hyperosmolality stimulate hepatocyte growth factor secretion from cultured human mesangial cells. *Diabetologia* 37:533-535, 1994
 47. BRIGGS JP, SCHNERMANN J: Whys and wherefores of juxtaglomerular apparatus function. *Kidney Int* 49:1724-1726, 1996
 48. TANAKA T, GRESIK EW, MICHELAKIS AM, BARKA T: Immunocytochemical localization of renin in kidneys and submandibular glands of SWR/J and C57Bl/6J mice. *J Histochem Cytochem* 28:1113-1118, 1980
 49. CELIO MR, INAGAMI T: Angiotensin II immunoreactivity coexists with renin in the juxtaglomerular cells of the kidney. *Proc Natl Acad Sci USA* 78:3897-3900, 1981
 50. TOJO A, GROSS SS, ZHANG L, TISHER CC, SCHMIDT HH, WILCOX CS, MADSEN KM: Immunocytochemical localization of distinct isoforms of nitric oxide synthase in the juxtaglomerular apparatus of normal rat kidney. *J Am Soc Nephrol* 4:1438-1447, 1994
 51. SHEHATA M, COPE GH, JOHNSON TS, RAFTERY AT, EL NAHAS AM: Cyclosporine enhances the expression of TGF- β in the juxtaglomerular cells of the rat kidney. *Kidney Int* 48:1487-1496, 1995
 52. SHEHATA M, EL NAHAS AM, BARKWORTH E, COPE GH, RAFTERY AT: Localisation of PDGF-BB in the juxtaglomerular cells of cyclosporin-treated rats. *Exp Nephrol* 3:173-179, 1995
 53. HORIKOSHI S, MCCUNE BK, RAY PE, KOPP JB, SPORN MB, KLOTMAN PE: Water deprivation stimulates transforming growth factor-beta 2 accumulation in the juxtaglomerular apparatus of mouse kidney. *J Clin Invest* 88:2117-2122, 1991
 54. RAY P, MCCUNE B, GOMEZ R, HORIKOSHI S, KOPP J, KLOTMAN P: Renal vascular induction of TGF- β 2 and renin by potassium depletion. *Kidney Int* 44:1006-1013, 1993
 55. ANTONIPILLAI I, LE TH, SOCENEATU L, HORTON R: Transforming growth factor- β is a renin secretagogue at picomolar concentrations. *Am J Physiol* 265:F537-F541, 1993
 56. KAGAMI S, BORDER WA, MILLER DE, NOBLE NA: Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431-2437, 1994
 57. ANDERSON S, JUNG FF, INGELFINGER JR: Renal renin-angiotensin system in diabetes: Functional, immunohistochemical, and molecular biological correlations. *Am J Physiol* 265:F477-F486, 1993
 58. KRIZ W, BANKIR L: A standard nomenclature for structures of the kidney. *Kidney Int* 33:1-7, 1988
 59. KREISBERG JJ, VENKATACHALAM MA: Morphological factors in acute renal failure, in *Acute Renal Failure*, edited by BRENNER BL, New York, Churchill Livingstone, 1988, pp 45-65
 60. NUYTS GD, YAQOUB M, NOUWEN EJ, PATRICK AW, MCCLELLAND P, MACFARLANE IA, BELL GM, DE BROE ME: Human urinary intestinal alkaline phosphatase as an indicator of S3-segment-specific alterations in incipient diabetic nephropathy. *Nephrol Dial Transplant* 9:377-381, 1994
 61. IHM CG, LEE GS, NAST CC, ARTISHEVSKY A, GUILLERMO R, LEVIN PS, GLASSOCK RJ, ADLER SG: Early increased renal procollagen alpha 1(IV) mRNA levels in streptozotocin induced diabetes. *Kidney Int* 41:768-777, 1992
 62. RONCO P, POLLARD H, GALCERAN M, DELAUCHE M, SCHWARTZ JC, VERROUST P: Distribution of enkephalinase (membrane metalloendopeptidase, E.C.3.4.24.11) in rat organs. *Lab Invest* 58:210-217, 1988

63. STEPHENSON SL, KENNY AJ: The hydrolysis of alpha human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase 24.11. *Biochem J* 243:183-187, 1987
64. URA N, CARRETERO OA, ERDOS EG: Role of renal endopeptidase in kinin metabolism in vitro and in vivo. *Kidney Int* 32:507-513, 1987
65. ABASSI ZA, TATE JE, GOLOMB E, KEISER HR: Role of neutral endopeptidase in the metabolism of endothelin. *Hypertension* 20:89-95, 1992
66. TANK JE, MOE OW, STAR RA, HENRICH WL: Differential regulation of rat glomerular and proximal tubular renin mRNA following uninephrectomy. *Am J Physiol* 270:F776-F783, 1996
67. HARRIS MP, CHEN M, BRIGGS JP, SCHNERMANN, JB: Renin messenger RNA in rat proximal convoluted and straight tubules. (abstract) *FASEB J* 7:A631, 1993
68. ELTAYEB BO, ALZAHABI B, ZIYADEH FN, RHODE R, LEWIS EJ, SHARMA K: Effects of captopril on serum levels of TGF- β 1 in insulin-dependent diabetic patients. (abstract) *J Am Soc Nephrol* 8:110, 1997
69. LEWIS EJ, HUNSICKER LG, BAIN RP, ROHDE RD, FOR THE COLLABORATIVE STUDY GROUP: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. *N Engl J Med* 329:1456-1462, 1993