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Rat mesangial cell hypertrophy in response to transforming growth factor-β1

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Rat mesangial cell hypertrophy in response to transforming growth factor-β1. Central features of progressive glomerular sclerosis are initial glomerular hypertrophy and subsequent accumulation of extracellular matrix proteins. Since TGF-β1 may play a key role in this glomerular response to injury, the present study sought to explore further TGF-β1 actions and regulated expression of its receptor in rat mesangial cells. The rat TGF-β type II receptor (TGF-βRII) homolog was cloned by screening a rat kidney cDNA library with a human TGF-βRII cDNA probe, and sequenced. Expression of this receptor subtype in rat mesangial cells was then demonstrated by RNase protection assay, and by Northern blot analysis of poly (A)+ RNA, TGF-βRII expression was down-regulated in cells treated with exogenous TGF-β1. Affinity cross linking studies demonstrated presence of this receptor on cell surface. Rat mesangial cells also expressed TGF-β1 and autoinduction by TGF-β1 was observed in the same cells, suggesting that this polypeptide may act in an autocrine fashion on mesangial cells, and that it may stimulate a positive autoamplification loop. TGF-β1 inhibited mesangial cell proliferation and stimulated significant overall protein and collagen production. Furthermore, mesangial cell size increased in response to chronic TGF-β1 treatment. These findings demonstrate that rat mesangial cells express key components of the TGF-β system and raise the intriguing possibility that in the glomerular mesangium, TGF-β1 may not only induce extracellular matrix synthesis, but may also participate in the process of glomerular hypertrophy in response to injury.

It has been suggested that progressive glomerular sclerosis, commonly observed in renal diseases which advance to end-stage renal failure, represents a final common response to injury [1]. A prominent feature of progressive glomerular sclerosis is the deposition of extracellular matrix proteins with eventual obliteration of glomerular capillary tufts and the consequent cessation of filtration [2]. The mechanisms mediating glomerular sclerosis are only partially understood. In the experimental model of extensive renal ablation in the rat, the compensatory increase in single nephron glomerular filtration rate in remnant glomeruli is mediated partly by higher than normal single nephron plasma flow rates and intracapillary hydraulic pressures, and partly by glomerular hypertrophy [1]. This compensatory response appears to be maladaptive, in that it is associated with the development of progressive proteinuria and glomerular damage. Similarly, in experimental diabetes mellitus in the rat, hemodynamic alterations and early hypertrophy are associated with the development of progressive proteinuria, glomerular sclerosis and loss of glomerular function [3]. In patients, hypertrophy of more normal "remnant" glomeruli is commonly observed in renal diseases where inhomogeneous injury has resulted in a significant loss of functioning nephrons [4]. Also, in patients with diabetic nephropathy, renal and glomerular hypertrophy [5] and higher than normal glomerular filtration rates [6] are frequently observed prior to the development of significant proteinuria and glomerular sclerosis. Prevention of glomerular capillary hypertension in experimental models of glomerular sclerosis protects against the development of progressive glomerular damage, suggesting that glomerular capillary hypertension is causally linked to the development of glomerular sclerosis [7]. It has also been argued that glomerular hypertrophy, not glomerular capillary hypertension, represents the more important causative factor in this process [8]. Both mechanisms are probably related, and are likely to involve biochemical mediators that signal cell hypertrophy and extracellular matrix production.

With regard to the kidney, enhanced TGF-β1 expression and TGF-β1 bioactivity has been documented in a model of acute antithymocyte serum-induced glomerulonephritis [9] and in experimental antiglomerular basement membrane antibody-mediated crescentic glomerulonephritis [10]; excess matrix deposition was prevented in the former model with a neutralizing TGF-β1 antiserum [11]. These studies therefore support the view that TGF-β1 participates in the process of glomerular matrix deposition in some forms of acute glomerulonephritis. Findings by Border and Ruoslahti [12] furthermore suggest enhanced glomerular expression of TGF-β1 in experimental diabetes mellitus in the rat, and the same group has reported a marked decrease in TGF-β1 expression in acute experimental glomerulonephritis in response to a reduction in protein intake [13]. Taken together, these data suggest that TGF-β may be an important factor participating in the excess extracellular matrix deposition observed in a number of glomerular diseases.

Type β transforming growth factors (TGFs-β) are multifunctional mediators which may play a role in progressive glomerular sclerosis. Three distinct mammalian TGFs-β have been identified [14], TGF-β1 being the most abundant isoform, which are found in large quantities in platelets and bone, and produced by most cells studied so far. TGFs-β are released from cells and platelets in the latent form, latency being conferred by association of active TGF-β after its initial cleavage from the

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propeptide with two binding proteins derived from the TGF-β precursor, and with one other latency conferring protein [15, 16]. Activation of TGFs-β through proteolytic release of the latency conferring proteins is required prior to its interaction with cell-surface receptors [17, 18].

TGFs-β regulate diverse cellular functions, including proliferation, differentiation and developmental processes [19]. In many systems, the TGFs-β inhibit cell proliferation, findings which have led to the theory that disruption of TGF-β actions may result in unrestrained proliferation of cells [16]. Indeed, tumor forming potential by some mammary epithelial cells appears to be related to a failure of TGF-β receptor expression [20, 21]. By contrast, enhanced expression of TGF-β action has been observed in wound healing, in the repair of vascular injury [22], and in diseases associated with interstitial fibrosis, such as pulmonary fibrosis [23], hepatic cirrhosis [24], and in the skin lesions of systemic sclerosis [25]. Previous studies also implicate TGF-β in the process of enhanced glomerular matrix deposition [26]. In this regard, it is of importance that TGFs-β potently stimulate synthesis of a number of extracellular matrix proteins by a variety of cells [26, 27], including glomerular mesangial cells [28, 29], and blunt mechanisms involved in extracellular matrix degradation [30, 31].

TGFs-β bind to specific cell surface receptors which mediate their actions. Two TGF-β receptor subtypes with a reasonably well-established role in signaling cell responses have been described, namely a ~60 kDa protein (the type I receptor) and a ~85 kDa protein (the type II receptor) [21]. A third receptor subtype, the type III receptor, is a membrane anchored proteoglycan with a very short intracytoplasmic domain, which is not thought to evoke a direct cell signaling response [32]. The type IV receptor has been identified by radioligand binding studies in rat pituitary cells, and unlike the other TGF-β receptors, it appears also to bind activin and inhibin, which are considered distantly related to TGF-β1 as members of the large TGF-β family. The biological significance of this type IV receptor remains to be determined [33]. Recently, the high molecular weight (~400 kDa) type V receptor has been identified and purified from bovine liver plasma membranes, and demonstrated to have a serine/threonine kinase activity [34]. The types I, II, III, and V receptors co-express in most normal cell types [35]. Based principally on information derived from cells not expressing types I or II receptors, both of the smaller cell-surface receptors appear to be involved in TGF-β1 signal transduction [21, 36], though the mechanism of TGF-β1 signaling in cells was obscure until recently. Sequence information for the type II receptor (TGF-βRII) was recently obtained after expression cloning of its cDNA in COS cells [21]. Based on the predicted amino acid sequence and on expression data, the TGF-βRII is a membrane-bound protein with a single transmembrane spanning domain which binds TGF-β1 in a rather short extracellular domain. The cytoplasmic domain of this receptor contains sequence highly homologous with the serine/threonine kinase family [21], though activation of kinase activity by ligand binding to the TGF-βRII has yet to be demonstrated. The receptors I and II associate as interdependent components of a heteromeric complex: receptor I requires receptor II to bind TGF-β, and receptor II requires receptor I to signal [37].

Given the potential importance of TGF-β in the process of the glomerular response to injury, the present study sought to further define TGF-β actions in mesangial cells. Because progressive glomerular sclerosis is most commonly studied in rat models, the nucleotide sequence for the rat homolog of TGF-βRII was first established. The study further demonstrates down-regulation of TGF-βRII expression by exogenous TGF-β1 and presence of the TGF-βRII protein on the cell surface, autoinduction of TGF-β1 expression and TGF-β1-induced hypertrophy in rat glomerular mesangial cells. The findings raise the intriguing possibility that TGF-β1 might play a role in the process of glomerular hypertrophy in response to injury.

Methods

RNA isolation and Northern blot hybridization

To obtain rat kidney RNA, whole kidneys from rats 1 day of age were homogenized in 5.5 M guanidine isothiocyanate (GTC); total RNA was isolated by ultracentrifugation on a cesium-TFA gradient and poly (A)+ RNA recovered from oligo(dT)-cellulose. Pooled neonatal rat cDNA was prepared by reverse transcription of 5 μg of poly (A)+ RNA with Superscript RT (GIBCO BRL). Total RNA from cultured rat mesangial cells and human retinal epithelial cells (provided by V. Reddy) were obtained by the same method described above.

For Northern blot analysis (Fig. 3), 3 μg poly (A)+ RNA from rat mesangial cells incubated for 24 hours in the presence or absence of 3 ng/ml TGF-β1 purified to 95% homogeneity by SDS-PAGE from porcine platelets (R&D Systems Inc., Minnesota, USA) was isolated and size fractionated on a 1% agarose-0.37 M formaldehyde gel in 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA, pH 7.0, transferred to a nylon membrane in 20× SSC (1× SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and UV-crosslinked. Hybridization was done at high stringency in 5× SSPE, 10 mM Tris-HCl, pH 8.0, 50% formamide, 2.5× Denhardt's solution, 100 μg/ml salmon sperm DNA at 42°C overnight, followed by one 20 minute wash at room temperature with 2× SSC, 0.1% SDS and two 30 minute washes at 60°C with 0.2× SSC, 0.1% SDS. The cloned rat TGF-βRII cDNA probe was labeled using the random primer labeling system (GIBCO BRL). Aldolase probe was a full-length 1.4 kb cDNA from ATCC (Rockville, Maryland, USA). The autoradiographs were analyzed by laser densitometer (Molecular Dynamics, Sunnyvale, California, USA).

Ten μg of total RNA from mesangial cells were used for the Northern analysis in Figure 5. The TGF-β1 probe was a 652 bp Kpn I cDNA fragment obtained from a full-length 2.14 kb TGF-β1 cDNA (ATCC) and subcloned into the pSPORT vector. The β-actin probe was a full-length 2.0 kb human β-actin cDNA obtained from Clontech.

Polymerase chain reaction

PCR primers for the kinase domain of the recently cloned human TGF-βRII [21] were prepared: sense primer: 5′-CCGGGATCCGTGGCAGTCAAGATCTTT-3′, antisense primer: 5′-CCGCTGCAGGCTCTTGAGGTCCCTGTG-3′. Total RNA (3 μg) from the cultured human retinal epithelial cell was reverse transcribed and the first strand cDNA was used for amplification. To obtain sequence for the rat TGF-βRII the

same primers were used, and pooled cDNA > 1 kb in size from rat neonatal kidney served as the template for PCR. For amplification, 10 μl of cDNA was combined with 87 μl PCR Mix: (10 μl 10× PCR buffer (Perkin-Elmer); 8 μl 2.5 mM dNTPs, 10 μl of 10 mM $MgCl_2$ and 59 μl H_2O) containing 0.25 μM (in 1 μl) of each PCR primer. The sample was then placed into the PCR cycler and heated for five minutes to 94°C followed by a temperature drop to 55°C. 2 U Taq polymerase were then added with a 50 μl mineral oil overlay, followed by 30 temperature cycles each consisting of three periods as follows: denature: 94°C for 45 seconds; anneal: 55°C for 60 seconds; extend: 72°C for 60 seconds. Following PCR, the products were separated on a 2% agarose gel. Products of the appropriate size from both human and rat templates were subcloned into the TA cloning vector pCR (Invitrogen) and sequenced using the dideoxy chain termination method. The sequence of one of the human clones was found to be identical to a 327 bp stretch in the kinase domain of the published human TGF-βRII cDNA [21].

Cloning and sequencing of the rat TGF-βRII cDNA

To construct a cDNA library, 5 μg of poly (A)+ RNA from neonatal rat kidney were reverse transcribed using Superscript RT (BRL). The library was generated in pSPORT1 (GIBCO BRL) from cDNA >1 kb in size. Blots from 5×10^5 independent colonies were screened using the partial human TGF-βRII cDNA previously derived by PCR as the probe. Hybridization was done in 5× SSPE, 10 mM Tris-HCl, pH 8.0, 40% formamide, 2.5× Denhardt's solution, and 100 μg/ml salmon sperm DNA at 42°C overnight, followed by one 20 minute wash at room temperature with 2× SSXC, 0.1% SDS and two 30 minute washes at 68°C with 0.2× SSC, 0.1% SDS. Three positive clones were identified and restriction analysis was done. For sequencing, one clone was isolated and contained a cDNA approximately 3.0 kb in length. Partially overlapping nested deletion clones were prepared from the cDNA with the Erase-a-Base kit (Promega), followed by sequencing of the clones using the dideoxy chain termination technique [38]. Ambiguous regions in the sequence were resolved by sequencing appropriate antisense strands.

Solution hybridization/RNase protection

RNase protection analysis was done using the RPA II kit (Ambion) according to the manufacturer's instructions. Briefly, the ^{32}P-labeled antisense RNA probe was prepared from the linearized plasmid containing a fragment of the newly cloned rat TGF-βRII cDNA using T7 RNA polymerase, yielding a probe 423 nucleotides long. The probe contains rat TGF-βRII antisense sequence 318 nucleotides in length, the remainder representing vector antisense sequences at each end. Six, 80 and 10 μg of total RNA from human retinal epithelial cells, rat mesangial cells and neonatal rat kidney, respectively, were hybridized with 1 to 4×10^5 cpm of the ^{32}P-labeled probe. Hybridization was for 16 to 18 hours at 42°C in 50% formamide, 5× SSPE, 0.1 M Tris, pH 7.4 and 50 μg/ml salmon sperm DNA. The samples were then digested with RNase A/T1, and resolved on a 6% acrylamide/7.7 M urea sequencing gel. A sample of ^{35}S-labeled DNA previously subjected to sequencing by the dideoxy chain termination method was loaded in adjacent lanes as the molecular size marker.

Covalent labeling of TGF-β receptors

Rat mesangial cells were incubated for three hours at 4°C in binding assay buffer [Hanks' balanced salt solution (HBSS), 40 mM Hepes (pH 7.4), 0.2 g/dl bovine serum albumin (BSA) and 1 mM bacitracin] with 400 pM ^{125}I-TGF-β1 (Collaborative Biomedical Products) in the presence and absence of 100 nM unlabeled TGF-β1. The cells were then washed twice with 40 mM Hepes (pH 7.4) in HBSS followed by incubation for one hour at 4°C with covalent cross-linking reagent ethylene glycol bis (succinimidyl succinate) in dimethyl sulfoxide at a final concentration of 2.5 mM in HBSS. The cross-linking reaction was quenched by the addition of an equal volume of 400 mM EDTA, 1 M Tris (pH 6.8). The cells were detached with a rubber policeman, transferred to a test tube and washed twice by centrifugation with 40 mM Hepes (pH 7.4) in HBSS at 4°C. The resulting pellet was resuspended in 100 μl of sample buffer [62.5 mM Tris base, 5% SDS, 5% glycerol, 0.01% bromphenol blue, ±2% β-mercaptoethanol, 5 mM EDTA (pH 6.8)], boiled for five minutes and subjected to centrifugation at 13,000 × g for 30 minutes to remove particulate matter, followed by 10 to 20% gradient SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (Bio-Rad) to visualize the molecular weight markers, and destained prior to autoradiography.

Cell culture

To culture rat glomerular mesangial cells, glomeruli were isolated from the renal cortex of 75 to 100 g female Sprague-Dawley rats using standard sieving techniques, digested at 37°C for 45 minutes with collagenase, 1 mg/ml (CLS 3, Worthington) in HBSS, washed three times by centrifugation at 800 × g, resuspended in RPMI 1640 medium containing 15% supplemented calf serum (Hyclone, Utah, USA), plated on 100 mm plates (Corning) and incubated in a humidified atmosphere of 5% CO_2 in air, at 37°C. The medium was changed every 48 hours. After 14 to 21 days in primary culture the cells were detached from the plates with trypsin/EDTA (GIBCO BRL) and replated at a 1:4 dilution. Subsequent passages were performed every 10 to 14 days, when the cells were still subconfluent. Cells between passages 3 and 6 were used for experiments described herein.

[^{3}H]Thymidine incorporation

Cells were plated in 24-well dishes and incubated in medium containing 15% SCS for five days. On day 6 the cells were fed with fresh medium containing 2% serum in the presence of increasing concentrations (0.03 to 3 ng/ml) of TGF-β1. Wells treated similarly but not containing TGF-β1 served as controls. After 45 hours, the medium was removed, and cells were exposed for three hours to [^{3}H]thymidine 1 μCi/ml in RPMI 1640 medium containing 2% SCS. The supernatant was removed, and cells were extracted three times with ice-cold 6% trichloroacetic acid (TCA). The cells were then washed once with water-saturated ether, allowed to dry, and solubilized in 0.1 N NaOH. The solubilized material was counted in a Packard liquid scintillation counter with 50% counting efficiency for [^{3}H].

A

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(-251)                                cgcgcgcgcacacgctcgcctgggggacggagccccagcctcctgcgcagctctcctcggccgccgggggc
(-180) ctcctccgggcctccgagctccggggatcgccggccacatctggcccgcacctctgcccagcgcgctccgggcggcgcaggcatcctgag
 (-90) agggcgaggaataaaggcgcagcccggggtccccgaggctcggttcgcggcgccccaggggccggtctatgacgagcgacgggggctgcc

  (1) ATGGGTCGGGGGCTGCTCCGGGGCCTGTGGCCGCTGCACATCGTCCTGTGGACGCGCATCGCCAGCACGATCCCGCCGCACGTTCCCAAG
      M  G  R  G  L  L  R  G  L  W  P  L  H  I  V  L  W  T  R  I  A  S  T  I  P  P  H  V  P  K  (30)

 (91) TCGGTTAACAGCGATCTGATGGCGGGTGACAACAGCGGTGCGGTCAAGCTTCCACAGCTGTGCAAGTTTTGCGACGTGACACTGTCCACT
      S  V  N  S  D  L  M  A  G  D  N  S  G  A  V  K  L  P  Q  L  C  K  F  C  D  V  T  L  S  T  (60)

 (181) TGTGACAACCAGAAGTCTTGCATGAGCAACTGCAGCGTCACCTCCATCTGTGAGAAGCCGCAGGAAGTCTGCGTGGCCGTGTGGAGGAAG
       C  D  N  Q  K  S  C  M  S  N  C  S  V  T  S  I  C  E  K  P  Q  E  V  C  V  A  V  W  R  K  (90)
                                *  *  *

 (271) AACGACAAGAACATTACTCTGGAGACCGTTTGCCACGACCCCAAGTTCACCTACCACGGCTTCACTCTGGAAGATGCCACTTCTCCCACG
       N  D  K  N  I  T  L  E  T  V  C  H  D  P  K  F  T  Y  H  G  F  T  L  E  D  A  T  S  P  T  (120)
                *  *  *

 (361) TGCGTCATGAAGGAAAAGAAAAGGGCGGGCGAGACCTTCTTCATGTGCTCCTGTAACACAGAGGAGTGTAACGATTACATCATCTTTAAT
       C  V  M  K  E  K  K  R  A  G  E  T  F  F  M  C  S  C  N  T  E  E  C  N  D  Y  I  I  F  N  (150)

 (451) GAAGAATACACCACCAGCAGTCCTGACCTGTTGCTGGTCATTATCCAAGTGACGGGCGTCAGCCTCCTGCCTCCGCTGGGGATTGCCATA
       E  E  Y  T  T  S  S  P  D  L  L  L  V  I  I  Q  V  T  G  V  S  L  L  P  P  L  G  I  A  I  (180)

 (541) GCTGTCATTGCCATCTTCTACTGTTACCGTGTCCACCGGCAGCAGAAGTTGAGCCCCTCCTGGGAGAGCAGCAAGCCCCGGAAGCTCATG
       A  V  I  A  I  F  Y  C  Y  R  V  H  R  Q  Q  K  L  S  P  S  W  E  S  S  K  P  R  K  L  M  (210)

 (631) GATTTCAGCGACAACTGCGCCATCATCCTGGAGGACGACCGCTCTGACATCAGCTCTACGTGCGCCAACAACATCAACCACAATACGGAA
       D  F  S  D  N  C  A  I  I  L  E  D  D  R  S  D  I  S  S  T  C  A  N  N  I  N  H  N  T  E  (240)

 (721) CTGCTGCCCATCGAGCTGGACACGCTGGTGGGGAAGGGCCGCTTCGCCGAGGTCTACAAGGCCAAGCTGAAGCAGAACACTTCAGAGCAG
       L  L  P  I  E  L  D  T  L  V  G  K  G  R  F  A  E  V  Y  K  A  K  L  K  Q  N  T  S  E  Q  (270)

 (811) TTTGAGACCGTGGCTGTCAAGATCTTCCCCTACGAGGAATACTCCTCGTGGAAAACGGAGAAGGACATCTTCTCTGACATCAACCTGAAA
       F  E  T  V  A  V  K  I  F  P  Y  E  E  Y  S  S  W  K  T  E  K  D  I  F  S  D  I  N  L  K  (300)

 (901) CACGAGAACATCCTGCAGTTCCTGACGGCCGAGGAGCGGAAGACGGAGATGGGCAAGCAGTACTGGCTGATCACGGCGTTTCATGCTAAG
       H  E  N  I  L  Q  F  L  T  A  E  E  R  K  T  E  M  G  K  Q  Y  W  L  I  T  A  F  H  A  K  (330)

 (991) GGCAACCTGCAGGAGTACCTCACTAGGCACGTCATCAGCTGGGAGGACCTGCGGAAGCTGGGCAGCTCCCTGGCCCGGGGCATCGCCCAT
       G  N  L  Q  E  Y  L  T  R  H  V  I  S  W  E  D  L  R  K  L  G  S  S  L  A  R  G  I  A  H  (360)

(1081) CTGCACAGTGACCACACTCCTTGTGGGAGGCCCAAGATGCCCATTGTTCACAGGGACCTCAAGAGCTCTAACATCCTAGTAAGAAGCGAT
       L  H  S  D  H  T  P  C  G  R  P  K  M  P  I  V  H  R  D  L  K  S  S  N  I  L  V  R  S  D  (390)

(1171) CTAACCTGTTGCCTGTGTGACTTCGGGTTGTCCTTGGGCCTGAGCCCTACTCTGTCTGTGGATGACCTGGCCAACAGCGGGCAGGTGGGA
       L  T  C  C  L  C  D  F  G  L  S  L  G  L  S  P  T  L  S  V  D  D  L  A  N  R  G  Q  V  G  (420)

(1261) ACAGCGAGATACATGGCCCCGGAAGTTCTAGAATCCAGGATGAATTTGGAGAACATGGAGTCCTTCAAGCAGACGGATGTCTACTCCATG
       T  A  R  Y  M  A  P  E  V  L  E  S  R  M  N  L  E  N  M  E  S  F  K  Q  T  D  V  Y  S  M  (450)

(1351) GCTCTAGTACTCTGGGAAATGACGTCGCGCTGCAACGCTGTGGGAGAAGTGAAAGATTATGAGCCCCCGTTTGGTTCCAGAGTGCGGGAG
       A  L  V  L  W  E  M  T  S  R  C  N  A  V  G  E  V  K  D  Y  E  P  P  F  G  S  R  V  R  E  (480)

(1441) CACCCCTGTGTGGAGAGCATGAAAGACAACGTGCTGAGAGACCGAGGACGGCCTGAAATCCCCAGCTTCTGGCTCAACCACCAGGGCATC
       H  P  C  V  E  S  M  K  D  N  V  L  R  D  R  G  R  P  E  I  P  S  F  W  L  N  H  Q  G  I  (510)

(1531) CAGATCGTGTGTGAGACACTGACCGAGTGCTGGGACCACGACCCCGAGGCCCGTCTCACAGCCCAGTGTGTGGCAGAGCGCTTCAGTGAA
       Q  I  V  C  E  T  L  T  E  C  W  D  H  D  P  E  A  R  L  T  A  Q  C  V  A  E  R  F  S  E  (540)

(1621) CTGGAGCACCCAGATAGACTCTCCGGGAGGAGCTGCTCCCAGGAGAAGATTCCAGAAGACGGCTCCCTGAACACTACCAAATAGctttttt
       L  E  H  P  D  R  L  S  G  R  S  C  S  Q  E  K  I  P  E  D  G  S  L  N  T  T  K  Z        (567)

(1711) ctgggcaggctgggcaagactccggaagtccgtcctctaaccaaagaccagaggtcagcaggattgctttcctgactgatgcttctggaa
(1801) aaccaaggacttgctcccttcttccctag
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Fig. 1. A. *The nucleotide and deduced amino acid sequences of the cDNA encoding the rat TGF-βRII.* The nucleotides and amino acids are numbered at the beginning and at the end of each line, respectively. The open reading frame sequence is indicated by upper-case letters, with the +1 position nucleotide denoting the start codon (ATG). An in-frame stop codon (TGA) is noted upstream of the initiator (ATG), at positions −19 to −21. The flanking untranslated nucleotide sequences are shown by lower-case letters. The extracellular cysteine residues are circled, and the potential N-linked glycosylation sites are indicated by asterisks. The hydrophobic transmembrane spanning domain is underlined. Arrows enclose the cytoplasmic serine/threonine kinase domain. **B.** Alignment of the amino acid sequences of the rat TGF-βRII homolog with the known human and porcine sequences. The published porcine amino acid sequence is incomplete, and indicated by dashes. Asterisks denote the positions where the rat cDNA sequence contains two extra amino acid residues. The shaded areas indicate amino acid identity.

B

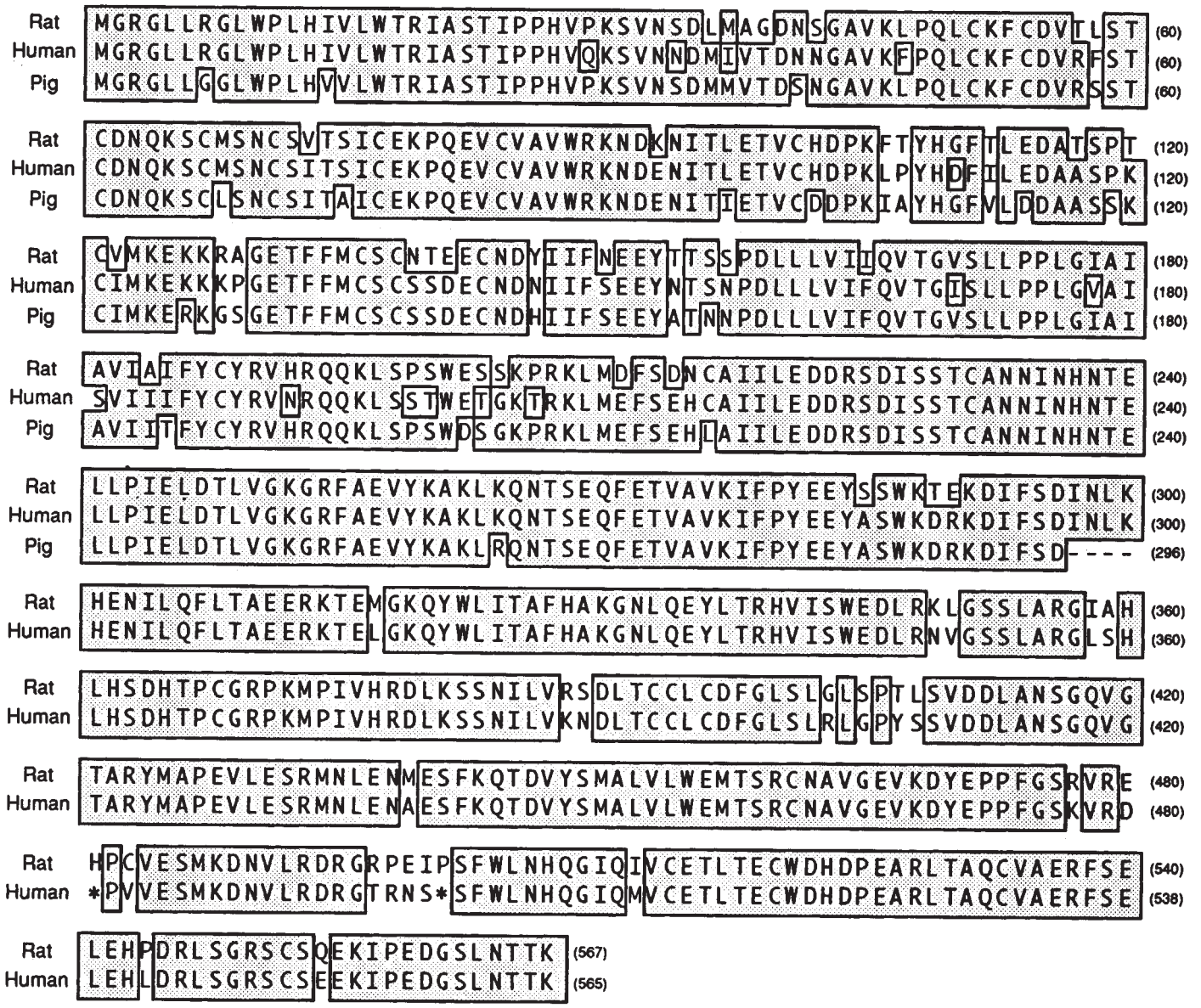

Fig. 1. *Continued*

[^{3}H]proline and [^{3}H]leucine incorporation

For [^{3}H]leucine and [^{3}H]proline incorporation, cells were treated as described above, except that only one concentration of TGF-β1, namely 3 ng/ml, was used. Cells not treated with TGF-β1 served as controls. [^{3}H]proline (2 μCi/ml, New England Nuclear, Boston, Massachusetts, USA) with 50 μg/ml ascorbic acid (Sigma) was added 24 hours after initiation of TGF-β1 treatment. After an additional 24 hours, cell and media proteins were precipitated with an equal volume of 12% ice-cold TCA. The cells were scraped from the plates and transferred together with the supernatant into 5 ml polystyrene tubes and the precipitated material was sedimented at 4°C and 1000 × g for 10 minutes. The material was washed three times with fresh 6% TCA and then solubilized in 2 ml of 0.2 N NaOH. A portion from each sample was subjected to liquid scintillation counting. The remainder was adjusted to contain NaCl 100 mM, Hepes 50 mM, $CaCl_2$ 3 mM, pH 7.4. Chromatographically-purified bacterial collagenase 100 U/ml (Type III, C0255 Sigma) was then added, followed by incubation for 12 hours at room temperature [27]. Following collagenase digestion the proteins were again TCA precipitated as described above, washed three times with ice-cold TCA, solubilized in 0.2 N NaOH and subjected to liquid scintillation counting. Collagenase-sensitive [^{3}H]proline incorporation was defined as the difference between TCA precipitable counts before and after collagenase digestion. On average, collagenase-sensitive [^{3}H]proline incorporation represented 50

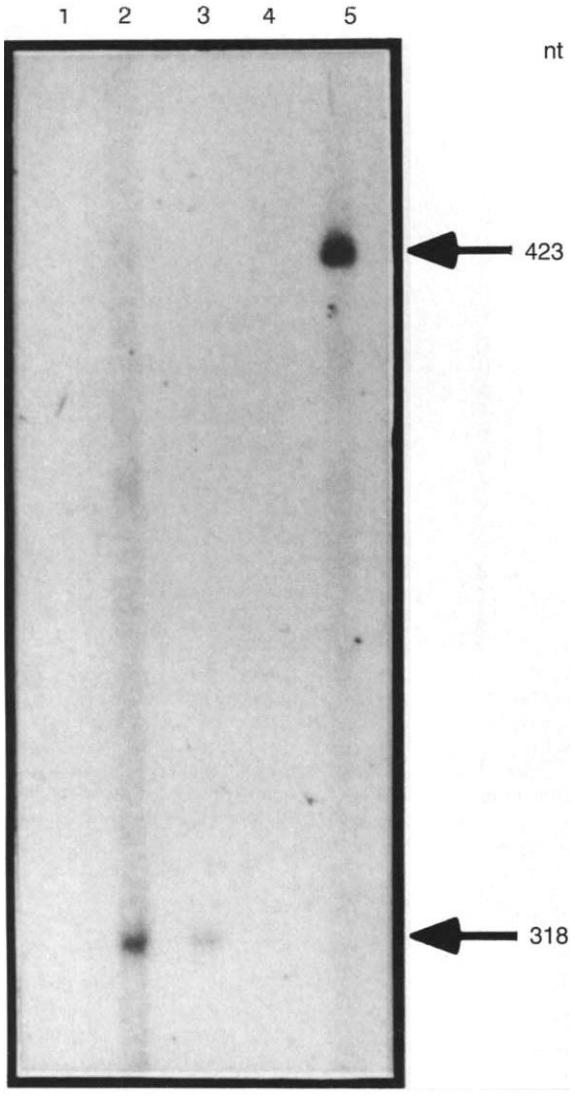

Fig. 2. *Expression of TGF-βRII in rat kidney and cultured rat mesangial cells.* Total RNA from cultured human retinal epithelial cells (lane 1), rat glomerular mesangial cells (lane 2), neonatal rat kidney (lane 3) was hybridized in solution with a ^{32}P-labeled rat TGF-βRII cDNA probe followed by RNase digestion. Controls represent ^{32}P-labeled rat TGF-βRII cDNA probe hybridized with tRNA, followed by digestion with RNase (lane 4), or without RNase treatment (lane 5). The rat TGF-βRII probe contained 318 nucleotides (nt) of authentic rat TGF-βRII sequence and as well as some vector sequence. The 318 nucleotides of authentic rat TGF-βRII were expected to hybridize fully with rat (but not human) TGF-βRII mRNA, the vector sequence was not expected to hybridize. Observed protected fragments were 318 nt in size (lanes 2 and 3), demonstrating that mRNA for rat TGF-βRII was present in total RNA from cultured rat mesangial cells and from rat kidney. As expected, no protected fragment was observed when the probe was hybridized with total RNA from human cells (lane 1) or with tRNA (lane 4). The undigested probe (containing flanking vector sequence) was 423 nt long (lane 5). Molecular size was determined from a concurrently run cDNA sequencing reaction.

to 60% of total proline incorporation; the fraction of collagenase-sensitive material did not change with TGF-β treatment.

[^{3}H]Leucine (1 μCi/ml) was added to the cells 40 hours after initiation of TGF-β treatment. At the end of an additional eight-hour incubation, the supernatant was removed, the cell proteins precipitated on the plate with ice-cold 6% TCA and washed three times with fresh TCA. The cells were then washed once with water-saturated ether, dried, solubilized in 1 N NaOH and subjected to scintillation counting. For all experiments, cell numbers were determined in replicate plates. [^{3}H]Proline and [^{3}H]leucine incorporation were corrected for cell number and are shown as the percent increase over incorporation in cells not treated with TGF-β1.

Cell proliferation and relative cell size determination

Mesangial cells were incubated in the presence of 2% supplemented calf serum with or without 3 ng/ml of TGF-β1 for 12 days. On days 1, 3 and 12 the cells were washed three times with phosphate buffered saline (PBS), detached from the plates with trypsin/EDTA, titurated to obtain a single cell suspension and counted in a Coulter Counter (Hialeah, Florida, USA). On day 12, cells similarly treated from 100 mm plates were also washed, detached and resuspended at a density of 10^6 cells/ml in PBS containing 2% SCS. The cells were then subjected, without fixation, to flow cytometry with an Epics C analyzer (Coulter, Epics Div.). Relative cell size for 5000 cells in each sample was determined by quantification of forward light scattering.

Results

TGF-βRII cDNA

A human TGF-βRII cDNA fragment 327 bp in length was obtained by PCR, subcloned into pCR and sequenced. The nucleotide sequence for this cDNA fragment was identical to the appropriate segment of the published sequence for the human TGF-βRII [21]. A rat cDNA fragment 318 bp in length was obtained by PCR amplification of neonatal rat kidney cDNA. A full-length rat TGF-βRII cDNA was cloned by screening the neonatal rat kidney library. The nucleotide sequence including the flanking noncoding regions, and the deduced amino acid sequence of this cDNA are shown in Figure 1A. An open reading frame of 1701 nucleotides was identified, corresponding to a protein of 567 amino acid residues. An in-frame stop codon (TGA) was noted upstream of the initiator (ATG). The predicted rat TGF-βRII protein contains twelve extracellular cysteine residues which define the Ig-like domains, which are 100% conserved between the species. Two potential N-linked glycosylation sites are identified in the extracellular domain. At the level of the nucleotide sequence, there is 85% sequence identity with the known human sequence, and 88% amino acid sequence identity. The published portion of porcine amino acid sequence has 84% homology with the rat TGF-βRII. The predicted protein encoded by the rat TGF-βRII has a single hydrophobic transmembrane spanning domain. The rat sequence contains two extra amino acids in the intracellular serine/threonine kinase domain. Based on the predicted amino acid sequence, the calculated molecular mass for the protein is 64 kDa. Figure 1B illustrates the degree of homology between the rat, human and pig sequences.

Regulated expression of TGF-βRII

Solution hybridization/RNase protection was performed using antisense RNA prepared from the rat TGF-βRII cDNA with

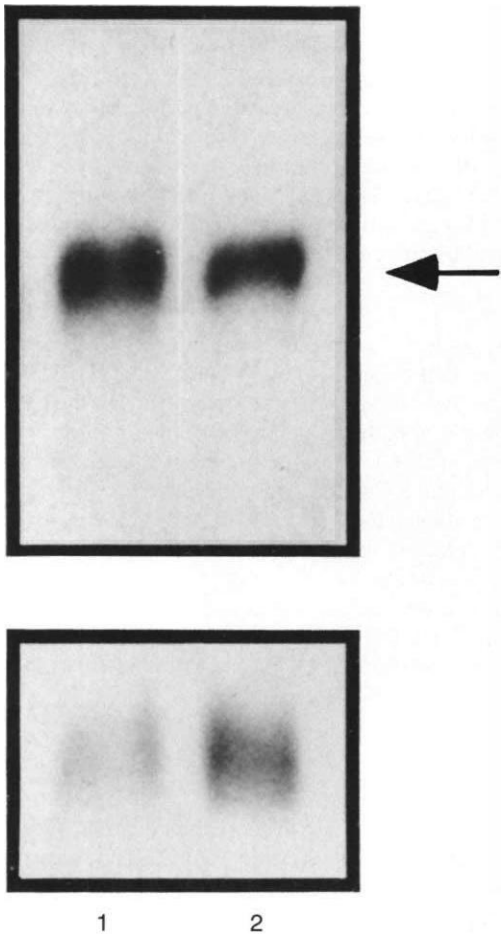

Fig. 3. *Down-regulation of TGF-βRII mRNA expression by TGF-β1 in mesangial cells.* Rat mesangial cells were incubated in the presence or absence of TGF-β1 (3 ng/ml) for 24 hours. Poly (A)+ RNA was isolated and 3 μg/lane was subjected to Northern blot hybridization with the cloned rat TGF-βRII cDNA probe (upper panels) or with a human aldolase probe (lower panels). A 5.5 kb mRNA (—▶) hybridized specifically with the TGF-βRII probe. By laser densitometry, TGF-βRII mRNA abundance was 2332 and 1713 arbitrary units for control and TGF-β1 treated cells, respectively. The abundance of aldolase mRNA was greater in TGF-β1 treated cells compared to controls (1179 vs. 1992 units).

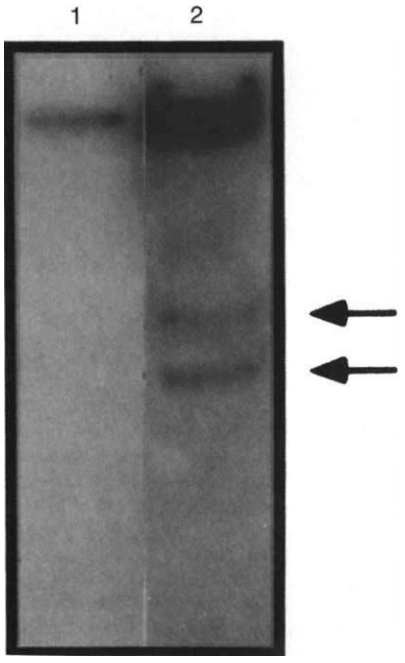

Fig. 4. *Covalent labeling of TGF-β receptors.* Affinity cross linking of ^{125}I-TGF-β1 to receptors on rat mesangial cells and separation by SDS-PAGE in the presence (lane 1) or absence (lane 2) of 100 nM unlabeled TGF-β1. Two specifically labeled bands are observed at approximately 60 kDa (lower —▶) and approximately 85 kDa (upper —▶) corresponding to the TGF-β type I and II receptors, respectively. The heavy labeling of material at the top of the gel likely represents type III proteoglycan TGF-β binding sites.

some flanking vector sequence. Based on the size of the TGF-βRII cDNA probe, the expected size of the protected fragment was 318 nucleotides. As shown in Figure 2, hybridization with the rat TGF-βRII antisense RNA probe protected a fragment 318 nucleotides in length from RNase digestion. These data therefore demonstrate expression of TGF-βRII mRNA in cultured rat mesangial cells.

To determine whether the TGF-βRII expression in rat mesangial cells was regulated by TGF-β1, the cells were treated with and without exogenous TGF-β1 (3 ng/ml) for 24 hours. Northern blots containing poly (A)+ mRNA from these cells (Fig. 3) demonstrated decreased TGF-βRII mRNA abundance in cells treated with TGF-β1. By densitometry, the signal strength for TGF-βRII mRNA was reduced ~30% in cells treated with TGF-β1, whereas that for aldolase increased ~40%. In studies not shown, β actin mRNA abundance also increased in cells treated with TGF-β1. Thus, whereas mRNA abundance for aldolase, β-actin and TGF-β1 (see below) increased in cells treated with TGF-β1, mRNA abundance for TGF-βRII decreased. Down-regulation of TGF-βRII mRNA was also observed in glomerular endothelial cells treated with exogenous TGF-β1 (data not shown).

Identification of receptors with affinity for TGF-β

Affinity cross-linking studies with ^{125}I-TGF-β1 in rat mesangial cells detected two distinct bands with molecular masses of approximately 85 and 60 kDa corresponding with TGF-βRII and TGF-β type I receptor, respectively (Fig. 4). The heavy labeling at the top of the gel likely represents Type III proteoglycan TGF-β binding sites.

Autoinduction of TGF-β1 mRNA in mesangial cells

On Northern blots, the TGF-β1 cDNA probe hybridized exclusively with a 2.4 kb mRNA. Under basal conditions, mesangial cells expressed the TGF-β1 mRNA, though mRNA abundance was exceedingly low. However, treatment of rat mesangial cells for as little as five hours with exogenous TGF-β1 (2 ng/ml) resulted in a significant augmentation of TGF-β1 mRNA abundance in the cells (Fig. 5).

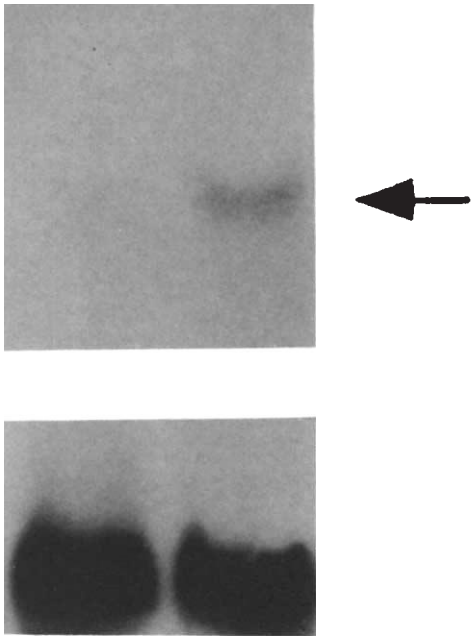

Fig. 5. *Autoinduction of TGF-β1 mRNA by TGF-β1 in mesangial cells.* Quiescent mesangial cells were treated with TGF-β1 (3 ng/ml) for 5 hours. Total RNA was then harvested from the cells, followed by Northern blot analysis with a human TGF-β1 cDNA probe (upper panels) or with a human β-actin probe (lower panels). Ten μg of total RNA was loaded in each lane. A 2.4 kb mRNA (—▶) hybridized specifically with the TGF-β1 cDNA probe. TGF-β1 mRNA abundance was greater in cells treated with TGF-β1 (lane 2) compared to cells left untreated (lane 1). Similar densities for the β-actin signals in both lanes indicate approximate equivalence of RNA loading.

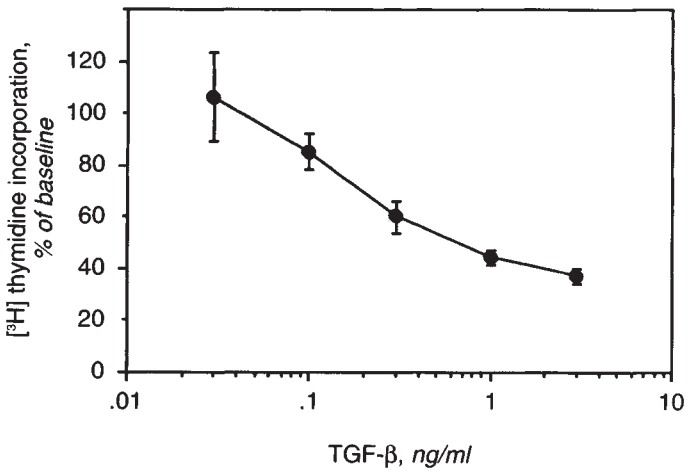

Fig. 6. *Inhibition of [3H]thymidine incorporation by TGF-β1 in cultured rat mesangial cells.* Mesangial cells grown in 2% serum were exposed to various concentrations of TGF-β1 for 45 hours, followed by [3H]thymidine incorporation into the cells for three hours. Under these conditions, TGF-β1 inhibited [3H]thymidine incorporation in a concentration-dependent fashion.

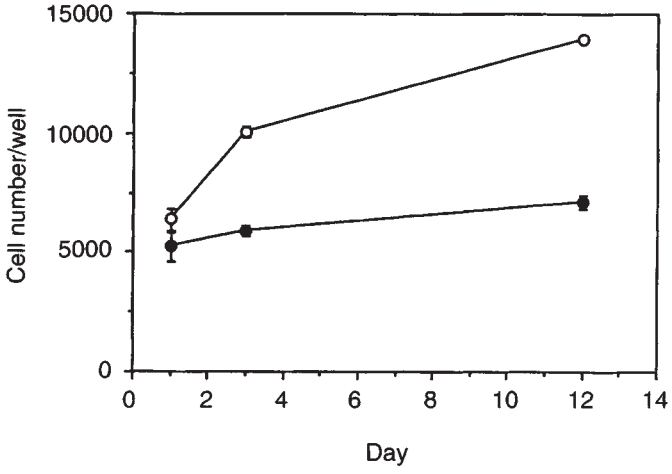

Fig. 7. *Inhibition of mesangial cell proliferation by TGF-β1.* Mesangial cells were grown in 2% serum for up to 12 days in the absence (○) or presence (●) of TGF-β1, 3 ng/ml. Media were changed every 48 hours. Data represent means of triplicate determinations ± SD for one representative experiment. Two other similar experiments gave essentially identical results.

Effect of TGF-β1 on mesangial cell [3H]thymidine incorporation and proliferation

As shown in Figure 6, treatment of mesangial cells (in 2% serum) for 48 hours with TGF-β1 significantly inhibited [3H]thymidine incorporation in a concentration dependent fashion by comparison with cells not treated with TGF-β1. An inhibitory effect was also observed in cells exposed to higher concentrations (15%) serum (data not shown). In these studies TGF-β1 had no inhibitory or stimulatory effect on DNA synthesis in quiescent mesangial cells cultured without serum (data not shown). Proliferation of mesangial cells was observed over 12 days when they were grown in 2% serum. Cell proliferation was almost completely inhibited in cells that were also exposed to exogenous TGF-β1 (3 ng/ml) during this culture period (Fig. 7).

Effect of TGF-β1 on mesangial cell [3H]proline and [3H]leucine incorporation

Whereas DNA synthesis and cell proliferation were inhibited by TGF-β1, there was a consistent and significant stimulation of protein synthesis, as determined by [3H]leucine incorporation (Fig. 8). During the first 48 hours of TGF-β1 treatment, total collagen synthesis, as determined by [3H]proline incorporation into collagenase-sensitive proteins, was augmented to a similar degree.

Effect of TGF-β1 treatment on mesangial cell size

Mesangial cells grown for 12 days in 2% serum in the absence or presence of exogenous TGF-β1 were subjected to quantification of forward light scattering, a measure of relative cell size, by flow cytometry. As shown in Figure 9, the mean cell size was significantly greater in cells treated with TGF-β1 compared to untreated cells. Mean forward light scattering averaged 266 ± 12 and 391 ± 13 units (mean ± SD, $N = 3$, $P < 0.01$ by Student's t-test).

Discussion

This study was undertaken to further clarify the potential role of TGF-β1 as a mediator in the glomerular response to injury.

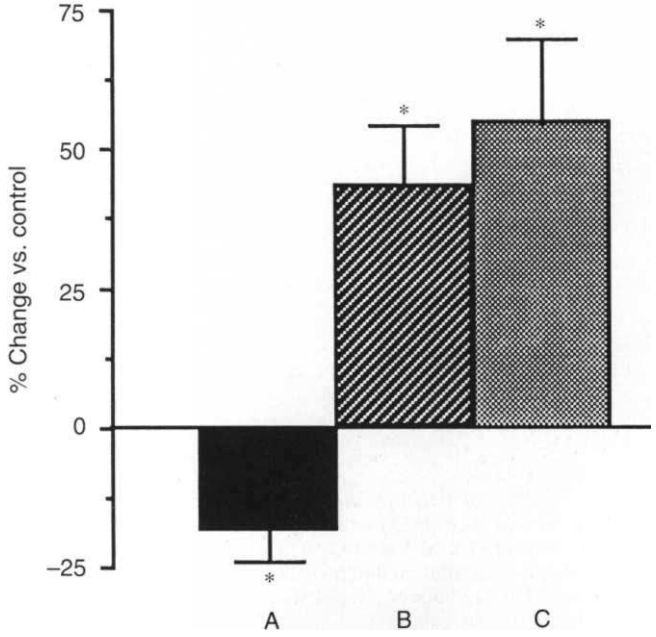

Fig. 8. *Stimulation of [^{3}H]leucine and [^{3}H]proline incorporation by TGF-β1.* Cell number (**A**), [^{3}H]leucine (**B**) and collagenase-sensitive [^{3}H]proline (**C**) incorporation were determined for mesangial cells cultured in 2% serum with or without TGF-β1 (3 ng/ml). Data for [^{3}H]leucine and [^{3}H]proline incorporation were corrected for cell number. Data for six separate experiments, each done in triplicate, are shown as the % change produced by TGF-β1, compared to cells not treated with TGF-β1 (means ± SEM, * $P < 0.01$, Student's *t*-test).

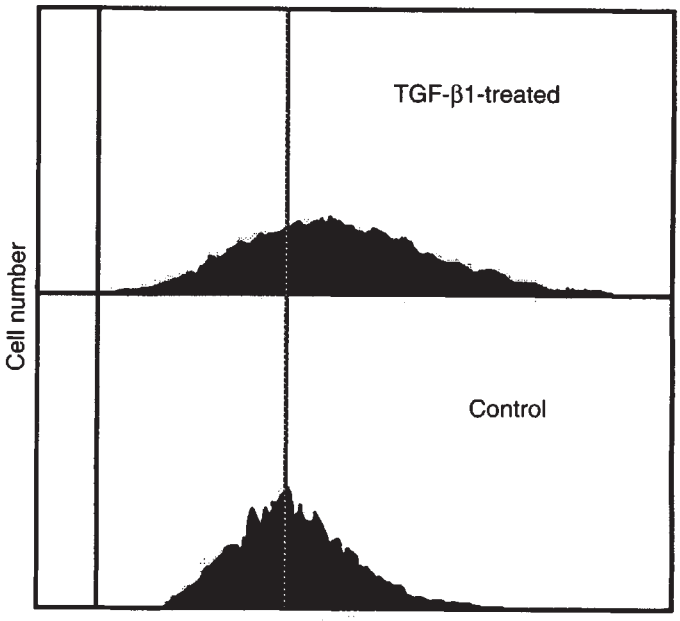

Fig. 9. *Mesangial cell hypertrophy in response to TGF-β1.* Rat mesangial cells grown under the same conditions as those shown in Figure 8 were detached from the plates and subjected to flow-cytometry. As a measure of cell size, forward light scattering (arbitrary units) was determined for a total of 5000 cells in each sample. The dotted vertical line corresponds to the mean cell size of cells not treated with TGF-β1 (control). One representative experiment is shown. Two other experiments gave essentially the same results.

The nucleotide sequence for a full-length rat TGF-βRII cDNA was first established. Rat glomerular mesangial cells in culture expressed mRNA for TGF-βRII as well as TGF-β1, and exogenous TGF-β1 significantly raised TGF-β1 mRNA abundance in mesangial cells. Exogenous TGF-β1 inhibited mesangial cell DNA synthesis and proliferation, but augmented collagen and overall cell protein synthesis in mesangial cells with a concomitant increase in mesangial cell size. The findings suggest that TGF-β1, in addition to its previously reported effects on extracellular matrix synthesis by mesangial cells [28, 29] may also participate in the process of glomerular hypertrophy, and furthermore, that this mediator may induce an autocrine amplification loop of TGF-β1 action through autoinduction of TGF-β1 expression.

Though receptors for TGF-β are widely expressed, not all cell types exhibit them on their cell surfaces [20, 21]. In murine mesangial cells high affinity binding sites for TGF-β receptors have been demonstrated, though the subtype of TGF-β receptor expressed by glomerular mesangial cells was not clearly established [28]. A human TGF-βRII cDNA was recently cloned and found to be a member of the receptor serine/threonine kinase family [21]. Using the published sequence information, human and rat TGF-βRII cDNA fragments were prepared by PCR and a full-length rat TGF-βRII homolog was cloned and sequenced. The rat TGF-βRII homolog shares 85% nucleotide sequence and 88% amino acid sequence identity with the known human TGF-βRII (Fig. 1 A and B). Rat species-specific sequence was used in this study to demonstrate TGF-βRII expression in mesangial cells by RNase protection assay and by Northern blot analysis. These data therefore indicate that mesangial cells in culture express the mRNA for the type II TGF-β receptor serine/threonine kinase. The rat-specific sequence should be useful for the study of TGF-βRII receptor expression and localization in rat models of glomerular disease. The finding that the TGF-βRII mRNA abundance in rat mesangial cells is reduced within 24 hours by exogenous TGF-β1 (Fig. 3) suggests that TGF-β1 negatively regulates expression of its own receptor at the level of transcription or mRNA stability. It is of interest that reduced TGF-βRII mRNA abundance was found in association with an overall increase in protein synthesis, and in association with increased expression of "housekeeping" genes like aldolase and β actin.

Affinity cross-linking studies (Fig. 4) detected two distinct cell surface receptors for TGF-β1 in rat mesangial cells corresponding to TGF-β type I and II receptors thought to be involved in the binding and signal transduction of TGF-β by association as a heteromeric complex [37]. Thus, not only was mRNA for TGF-βRII expressed in mesangial cells, but the receptor protein was also expressed at the cell surface.

To explore the biological actions of TGF-β1 on glomerular mesangial cells, evidence for TGF-β1 mRNA expression was also sought, and cells were examined for potential regulation of TGF-β1 mRNA abundance in response to TGF-β1 stimulation. By Northern blot analysis, TGF-β1 mRNA was expressed by rat mesangial cells, a finding supported by the recent report by Kaname et al [39]. In this study, TGF-β1 expression was at low

levels by quiescent mesangial cells. However, TGF-β1 mRNA abundance was significantly induced when the cells were treated with exogenous TGF-β1. These findings indicate that TGF-β1 mRNA is autoinduced by TGF-β1, suggesting that a positive amplification loop for TGF-β1 action is stimulated by TGF-β1 in these cells. Similar autoinduction of TGF-β expression has been demonstrated in human lung adenocarcinoma (A549) cells and has been attributed to two regions in the TGF-β promoter which are responsive to autoinduction and to phorbol ester and which bind the AP-1 transcription factor complex [40, 41]. However, given that TGF-β is secreted by most cells in the inactive form, autoinduction of TGF-β1 synthesis cannot necessarily be assumed to increase TGF-β1 action, since the regulation of TGF-β activity may not reside solely in its level of synthesis, but also at the level of activation/inactivation [42]. Nevertheless, the data in this study do indicate that TGF-β1 synthesis may be further augmented when the cells are treated with TGF-β1. If mesangial cells have the capability of activating TGF-β1, the findings suggest that the mediator could act in a purely autocrine fashion within the glomerular mesangium.

In mesangial cells grown in 2% serum, TGF-β1 inhibited [^{3}H]thymidine incorporation in a concentration-dependent manner, and inhibited cell proliferation. TGF-β1 has been shown to inhibit DNA synthesis and proliferation in response to serum and diverse growth factors in a number of cell types, an effect that may be related to dephosphorylation of the retinoblastoma gene product [43]. It has been suggested that this growth regulating mediator may play an important role in suppressing cell proliferation under basal conditions *in vivo*, and absence of TGF-β receptors and TGF-β suppression of growth have been postulated to enhance tumorigenic potential in some tumors of epithelial cell origin, such as mammary carcinoma [20, 43]. However, TGF-β1 can also stimulate proliferation of cells, and can act synergistically with other growth factors to induce the transformed phenotype [44]. In murine mesangial cells, the effect of TGF-β1 on proliferation was dependent on cell density: at higher cell densities the mediator stimulated growth, and at lower cell densities it was found to be inhibitory [28]. It has been suggested that TGFs-β may function as a cellular switch, tending to stimulate or inhibit proliferation and other cell functions depending on the basal state of the cell [16]. It therefore is tempting to speculate that TGF-β may act to suppress proliferation of mesangial cells under conditions when these cells are stimulated to proliferate, as might occur in the presence of an acute proliferative glomerulonephritis, whereas under conditions in which the mesangial cell growth is suppressed the cells could be stimulated to grow.

A large number of prior publications have demonstrated enhanced production of extracellular matrix material by a wide variety of cells in response to TGF-β [27, 45]. Enhanced synthesis of fibronectin, heparan sulfate proteoglycans and of collagen by glomerular mesangial cells *in vitro* has been demonstrated [26, 29, 46]. In this study, overall collagen synthesis, as determined by [^{3}H]proline incorporation into collagenase-sensitive proteins, was significantly increased during the first 48 hours of exposure of the cells to TGF-β1. However, not only was there enhanced proline incorporation into collagen, but total protein synthesis rates were also augmented as indicated by enhanced [^{3}H]leucine incorporation (Fig. 8). Given the enhanced protein synthesis rates in the presence of suppressed cell proliferation, it was postulated that TGF-β1 must result in cellular hypertrophy. Indeed, when assessed by forward light scattering, mesangial cells treated with TGF-β1 for 12 days were found, on average, to be significantly larger than cells not treated with TGF-β1 (Fig. 9). These findings are consistent with the marked alteration in mesangial cell morphology in response to TGF-β1 observed by MacKay et al [28]. Furthermore, hypertrophy of vascular smooth muscle cells in response to TGF-β1 treatment has also been reported [47].

In summary, the nucleotide and amino acid sequences for the rat TGF-βRII were obtained; expression of the receptor in rat mesangial cells was then demonstrated by RNase protection and Northern blot analysis. TGF-β1 down-regulated TGF-βRII mRNA expression. Affinity cross-linking studies detected two distinct TGF-β receptors, namely type I and type II, on the surface of rat mesangial cells. The finding that TGF-β1 is also expressed and autoinduced by TGF-β1 in the same cells suggests that this polypeptide may act in an autocrine fashion on mesangial cells, and that it may stimulate a positive autoamplification loop. Though mesangial cell proliferation was inhibited by TGF-β1, a significant increase in protein and collagen production was found and overall cell size increased in response to TGF-β1 treatment. Taken together, these findings suggest that in the glomerular mesangium, the components of the TGF-β system are expressed in a regulated fashion, and that TGF-β1 not only affects extracellular matrix synthesis, but may also participate in the process of glomerular hypertrophy.

Acknowledgments

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