Inhibition of Capillary Morphogenesis and Associated Apoptosis by Dominant Negative Mutant Transforming Growth Factor-β Receptors*

Mary E. Choiñ and Barbara J. Ballermann

From the Division of Nephrology and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Transforming growth factor-β1 (TGF-β1) induces angiogenesis in vivo and capillary morphogenesis in vitro. Two receptor serine/threonine kinases (types I and II) have been identified as signal transducing TGF-β receptors. We explored the possibility of inhibiting TGF-β-mediated events in glomerular capillary endothelial cells using a TGF-β type II receptor (TβR-II) transdominant negative mutant.

A mutant TGF-β type II receptor (TβR-IIIM), lacking the cytoplasmic serine/threonine kinase domain, was produced by polymerase chain reaction using rat TβR-II cDNA as template. Since TβR-I and TGF-β type I receptor (TβR-I) heterodimerize for signal transduction, the mutant receptor competes for binding to wild-type TβR-I, hence acting in a dominant negative fashion. Glomerular capillary endothelial cells were stably transfected with TβR-IIIM, and four independent clones were expanded. That the TβR-IIIM mRNA was expressed was shown by reverse transcriptase-polymerase chain reaction, RNase protection assay, and Northern analysis. Presence of cell surface TβR-IIIM protein was shown by affinity cross-linking with 125I-TGF-β1. In wild-type endothelial cells, TGF-β1 (2 ng/ml) significantly inhibited [3H]thymidine incorporation to 63 ± 10% of control (n = 4). In transfected endothelial cells carrying TβR-IIIM, TGF-β1 stimulated [3H]thymidine incorporation to 131 ± 9% of control (n = 4, p < 0.005). Also, in wild-type endothelial cells, endogenous and exogenous TGF-β1-induced apoptosis and associated capillary formation. Both apoptosis and capillary formation were uniformly and entirely absent in transfected endothelial cells carrying TβR-IIIM.

This represents the first demonstration that capillary morphogenesis in vitro is associated with apoptosis, and that interference with TβR-II signaling inhibits this process in glomerular capillary endothelial cells.

Angiogenesis, the process of new blood vessel formation, is an integral part of development, wound repair, and tumor growth. The formation of capillary networks requires a complex series of cellular events, in which endothelial cells locally degrade their basement membrane, migrate into the connective tissue stroma, proliferate at the migrating tip, elongate and organize into capillary loops (1). In response to angiogenic stimuli, endothelial cells in culture develop networks of capillary-like tubes.

Transforming growth factor-β1 (TGF-β1) is a 25-kDa homodimeric polypeptide that belongs to a family of homologous multifunctional cytokines. TGF-β1 regulates diverse cellular functions including proliferation and differentiation. TGF-β1 is strongly expressed during embryogenesis (2, 3) and in sites undergoing intense development and morphogenesis (4, 5). Moreover, TGF-β1 induces angiogenesis in vivo (6, 7) and capillary morphogenesis in vitro (8). The mechanism by which TGF-β1 induces angiogenesis is not yet well defined.

In the early stages of angiogenesis, proteases are required for extracellular matrix (ECM) proteolysis to facilitate endothelial cell migration (9). TGF-β1 induces endothelial cell secretion of plasminogen activator (PA) which activates plasmin, a protease that degrades ECM proteins (10, 11). Increased production of PA has been associated with the invasive properties of cultured endothelial cells in response to angiogenic stimuli (10, 11). In addition, plasmin activates latent TGF-β1 (12), in an autocrine fashion. Furthermore, TGF-β1 is a potent chemoattractant for macrophages and fibroblasts (13, 14), which are postulated to release angiogenic peptides in vivo, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor, or tumor necrosis factor-α (15).

Two transmembrane serine/threonine kinases, types I and II, have been identified as signal transducing TGF-β receptors. TGF-β type II receptor (TβR-II), a constitutively active kinase, directly binds TGF-β1, and this ligand binding results in the recruitment and phosphorylation of TGF-β type I receptor (TβR-I) to produce a heteromeric signaling complex (16). TβR-I alone is unable to bind TGF-β1, and TβR-II is unable to signal without TβR-I (17).

We explored the possibility of inhibiting TGF-β1-mediated events in renal glomerular capillary endothelial cells using a TβR-II transdominant negative mutant. A mutant TβR-II construct (TβR-IIIM), lacking the cytoplasmic serine/threonine kinase domain, but with full transmembrane spanning and extracellular domains, was produced by polymerase chain reaction (PCR) using rat TβR-II cDNA (18) as template. Since TβR-II and TβR-I heterodimerize for signal transduction, the mutant receptor competes for binding to wild-type TβR-I, hence acting in a dominant negative fashion (19–21). When the trans-

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† Recipient of Physician Scientist Award 5-K12-DK0129809. To whom correspondence and reprint requests should be addressed: Div. of Nephrology, The Johns Hopkins University School of Medicine, Rm. 943, Ross Research Bldg., 720 Rutland Ave., Baltimore, MD 21205. Tel.: 410-614-0064; Fax: 410-955-0485.

1 The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; PA, plasminogen activator; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; TβR-II, TGF-β type II receptor; PCR, polymerase chain reaction; FBS, fetal bovine serum; nt, nucleotide; kb, kilobase(s); MOPS, 3-(N-morpholino)propanesulfonic acid; HBSS, Hanks’ balanced salt solution.
dominant negative mutant construct was stably expressed in glomerular capillary endothelial cells, capillary morphogenesis and associated apoptosis were entirely blocked in these cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Glomerular capillary endothelial cells were isolated from bovine kidney cortex as described previously (22), with the following modifications. After collagenase digestion, the cells were plated at low density on gelatin-coated plates, in RPMI 1640 medium containing 15% fetal bovine serum (FBS) to which 8 ng/ml acidic fibroblast growth factor (aFGF) (R & D Systems), 0.15% fetal bovine serum (FBS) to which 8 ng/ml acidic fibroblast growth factor (aFGF) (R & D Systems), was added to each well to make a final concentration of 10% FBS, and incubated further for 48 h. Then the medium was changed to 10% FBS in RPMI (no antibiotics) and incubated for another 24 h. To select for stable transfectants, cells were treated with 400 μg/ml Geneticin (Life Technologies, Inc.) in RPMI medium containing 15% FBS, and the medium was changed every 2-3 days. Clones emerged at approximately 14 days after lipofection. Stably transfected clones were subcloned using ring cylinders, expanded, and maintained in RPMI medium containing 15% FBS, 200 μg/ml Geneticin, 5 units/ml penicillin, and 5 μg/ml streptomycin. Four independent, stably transfected clones containing TjaR-IIw and 4 clones containing empty vector were expanded.

Non-transfected glomerular endothelial cells similarly treated with Lipofectin served as additional controls.

Solution Hybridization/RNase Protection—RNase protection analysis was done using the RPA II kit (Ambion) according to the manufacturer’s instructions. The 32P-labeled antisense RNA probe was prepared from the linearized plasmid containing a fragment of the rat TjaR-II cDNA using T7 RNA polymerase, yielding a probe 488 nucleotides (nt) long. 20 μg of total RNA from wild-type and transfected cells were hybridized with the 32P-labeled probe. Hybridization was for 16-18 h 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 32P-labeled 1-kb ladder DNA was loaded in adjacent lanes as the molecular size marker.

Northern Blot Analysis—Total RNA from cells grown in the absence or presence of 1 μM dexamethasone (Sigma) was isolated by lysis with TRI reagent (Molecular Research Center, Inc.) according to the manufacturer’s instructions, and size fractionated (30 μg/lane) on a 1% agarose, 2% formaldehyde gel in 20 mM MOPS, 5 mM sodium acetate, and 10 mM EDTA, pH 7.2. Messenger RNA was transferred to a nylon membrane (Schleicher & Schuell) and UV linked to the membrane. The blot was prehybridized at 65°C using 1% bovine serum albumin (Sigma), 7% SDS, 0.5 μM phosphatase buffer, 1 μM EDTA, pH 8.0, and 100 μg/ml heat-denatured salmon sperm DNA for 2 h, hybridized in the same solution containing the appropriate 32P-labeled cDNA at 65°C overnight, followed by two 30-min washes at 65°C with 0.5% bovine serum albumin, 5% SDS, 40 μM phosphatase buffer, 1 μM EDTA, pH 8.0, then four 15-min washes with 1% SDS, 40 μM phosphatase buffer, 1 μM EDTA, pH 8.0, at 65°C. The membrane was then exposed to Kodak X-AR 5 film for 25-48 h. The TjaR-II probe is a 2.8-kb rat TjaR-II full-length cDNA (17) which was labeled with [32P]dCTP using random primer labeling system (Life Technologies, Inc.).

Covalent Labeling of TGF-β Receptors—Cells on 100-mm plates (Corning) were washed twice with cold 40 μM HEPES, pH 7.4, in Hank’s balanced salt solution (HBSS), then incubated in binding assay buffer (HBSS, 40 μM HEPES, pH 7.4, and 1 μM bacitracin) with 400 μM 125I-TGF-β1 (DuPont) in the presence or absence of 100 nM unlabeled TGF-β1 (Collaborative Biomedical Products). At room temperature for 90 min. The cells were then washed twice with 40 μM HEPES, pH 7.4, in HBSS, followed by incubation for 30 min at 4°C with covalent cross-linking reagent disuccinimidyl carbonate (Pierce). The cross-linking reaction was quenched by washing three times with 250 μM succrose, 10 μM Tris, pH 7.4, 1 mM EDTA. The cells were lysed with 100 μl of 1% Triton X-100, 10 mM Tris, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 1 μM phenylmethylsulfon fluoride, 50 μg/ml aprotinin, 10 μg/ml pepstatin, and subjected to centrifugation at 13,000 × g for 30 min to remove particulate matter. Sample loading buffer (sucrose, 0.01% bromphenol blue, 2% β-mercaptoethanol, 5 mM EDTA) was added (1:1, v/v) and boiled for 5 min, followed by 10%
SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue (Bio-Rad) to visualize equivalence in protein loading, and destained prior to autoradiography.

\([^{3}H]Thymidine Incorporation—10^4 cells were plated in 24-well dishes and incubated in medium containing 15% FBS and grown to confluence. Medium was then changed to serum-free RPMI for 24 h, followed by incubation in 0.5% FBS in the presence or absence of TGF-\(\beta\)-1 (2 ng/ml). After 45 h, the medium was removed, and cells were exposed for 3 h to 1 \(\mu\)Ci/ml \([^{3}H]thymidine in RPMI 1640 medium containing 2% bovine platelet-poor plasma derived serum, at 37 \(^\circ\)C. The cells were washed three times with RPMI, and then extracted three times with ice-cold 6% trichloroacetic acid, followed by solubilization in 1 N NaOH and counted in a Packard Liquid scintillation counter. For determination of the time course of \([^{3}H]thymidine incorporation, similar methods were utilized and the cells were extracted at the various time points, with 3-h \([^{3}H]thymidine exposure prior to each period.

Genomic DNA isolation and Analysis—Cells were plated on 150-mm plates, and grown to confluence in medium containing 15% FBS, then incubated in the presence or absence of 1 \(\mu\)M dexamethasone for 24 h. The medium was then changed to 0.5% FBS and incubated in the presence or absence of TGF-\(\beta\)-1 (2 ng/ml) at 37 \(^\circ\)C for 5 days. Genomic DNA isolation was performed using Puregene (Gentra) according to the manufacturer’s instructions. Briefly, cells were lysed directly on the plate by removing the culture medium and adding the cell lysis solution, followed by incubation with RNase A, then protein precipitation solution. The samples were centrifuged at 2,000 \(g\) for 10 min and supernatant transferred to new tubes. Precipitated DNA was resuspended in DNA hydration solution, and quantitated by UV spectrophotometer. 20 \(\mu\)g of DNA was analyzed with a 1.5% agarose gel electrophoresis.

RESULTS

Expression of T\(\beta\)-II\(_M\) mRNA—To demonstrate that the transfected glomerular endothelial cells expressed T\(\beta\)-II\(_M\) mRNA, RNase protection assay was performed using antisense RNA prepared from the rat T\(\beta\)-II\(_M\) cDNA with some flanking vector sequence. The rat T\(\beta\)-II probe contained 488 nt of authentic rat T\(\beta\)-II sequence, which included only 211 nt of T\(\beta\)-II\(_M\), and predicted to hybridize fully with rat (but not bovine) T\(\beta\)-II mRNA. Based on the size of the T\(\beta\)-II cDNA probe, the expected size of the protected fragment produced from transfected rat T\(\beta\)-II\(_M\) cDNA sequence was 211 nt. As shown in Fig. 2A, hybridization with the rat T\(\beta\)-II antisense riboprobe protected a fragment 211 nt in length from RNase digestion in transfected cells. No protected fragment was seen in the wild-type bovine glomerular endothelial cells or mock transfected cells with vector alone. Expression of T\(\beta\)-II\(_M\) mRNA in glomerular endothelial cells was also demonstrated by RT-PCR utilizing the sense and antisense primers to rat T\(\beta\)-II and cDNA sequence used in producing the mutant construct (data not shown), and four independent transfected clones expressing T\(\beta\)-II\(_M\) mRNA were propagated for all subsequent experiments.

Northern blot analysis of total RNA isolated from wild-type and transfected glomerular endothelial cells, probed with T\(\beta\)-II cDNA, showed a 5.5-kb band in all cells (Fig. 2B), corresponding to wild-type T\(\beta\)-II. A 1.8-kb band, corresponding to T\(\beta\)-II\(_M\), was observed only in cells transfected with T\(\beta\)-II\(_M\), and not present in wild-type or mock transfected cells, and was strongly induced by dexamethasone. Also, a modest induction of the 5.5-kb wild-type T\(\beta\)-II mRNA was seen with dexamethasone.

Cell Surface Expression of T\(\beta\)-II\(_M\)—Affinity cross-linking with \(^{125}\)I-TGF-\(\beta\)-1 in wild-type glomerular endothelial cells detected two distinct bands with molecular masses of approximately 89 and 70 kDa corresponding with T\(\beta\)-II and T\(\beta\)-I, respectively (Fig. 3). Only in the transfected glomerular endothelial cells expressing T\(\beta\)-II\(_M\), two labeled bands of approximately 48 and 36 kDa were observed, both induced by dexamethasone. Labeling of T\(\beta\)-I in transfected glomerular endothelial cells appeared diminished in three separate experiments.

Effect of TGF-\(\beta\)-1 on Endothelial Cell \([^{3}H]Thymidine Incorporation—Treatment of wild-type glomerular endothelial cells in culture for 48 h with exogenous TGF-\(\beta\)-1 significantly inhibited \([^{3}H]thymidine incorporation to 63 \pm 10\% of control (Fig. 4A). Inhibition of \([^{3}H]thymidine incorporation was similarly observed in empty vector transfected cells treated with TGF-\(\beta\)-1. In contrast, treatment of transfected cells carrying T\(\beta\)-II\(_M\) with exogenous TGF-\(\beta\)-1 stimulated \([^{3}H]thymidine incorporation to 131 \pm 9\% of control (*, p < 0.005, Student's t test, n =

![Fig. 2. Expression of T\(\beta\)-II mRNA in wild-type and transfected glomerular endothelial cells. A, RNase protection assay. Total RNA from wild-type and transfected glomerular endothelial cells, treated with 1 \(\mu\)M dexamethasone, was hybridized in solution with a \(^{32}\)P-labeled rat T\(\beta\)-II cDNA probe followed by RNase digestion. As expected, no protected fragment was observed in the wild-type cells (lane 1). In the transfected cells (lane 2), a 211-nt long protected fragment was seen, demonstrating that mRNA for rat T\(\beta\)-II\(_M\) was expressed. Controls represent \(^{32}\)P-labeled rat T\(\beta\)-II cDNA probe hybridized with yeast total RNA, followed by digestion with RNase (lane 3), or without RNase treatment (lane 4). No protected fragment was seen with RNase treatment (lane 3). The undigested probe was 488 nt long (lane 4). Molecular size was determined by \(^{32}\)P-labeled 1-kb ladder DNA (not shown). B, Northern analysis. Total RNA (30 \(\mu\)g/lane) from wild-type glomerular endothelial cells incubated in the absence (lane 1) or presence (lane 2) of 1 \(\mu\)M dexamethasone was subjected to Northern blot hybridization with a \(^{32}\)P-labeled rat T\(\beta\)-II cDNA probe. Only a 5.5-kb mRNA was detected, corresponding to wild-type T\(\beta\)-II. Lanes 3 and 4 represent total RNA from transfected endothelial cells incubated in the absence or presence of 1 \(\mu\)M dexamethasone, respectively. Both the 5.5- and 1.8-kb mRNA are observed, reflecting wild-type T\(\beta\)-II and T\(\beta\)-II\(_M\) mRNA. The 1.8-kb mRNA was strongly induced by dexamethasone (lane 4). Also, a modest induction of the 5.5-kb wild-type T\(\beta\)-II mRNA was seen with dexamethasone (lanes 2 and 4). Similarly densities for the 18S signals indicate approximate equivalence of RNA loading.

![Fig. 3. Affinity cross-linking of \(^{125}\)I-labeled TGF-\(\beta\)-1 to cell surface receptors. Lanes 1 and 2 represent wild-type glomerular endothelial cells cross-linked in the presence or absence of unlabeled TGF-\(\beta\)-1, respectively. Lanes 3 and 4 represent transfected glomerular endothelial cells in the presence or absence of unlabeled TGF-\(\beta\)-1, respectively. Specifically labeled bands are observed at approximately 89 kDa and approximately 70 kDa corresponding to wild-type T\(\beta\)-II and T\(\beta\)-I, respectively. Bands of approximately 48 and 36 kDa are seen only in the transfected glomerular endothelial cells.
Endothelial cells carrying TGF-β1 of TGF-β1 and in the presence (lane E) or absence (lane D) of TGF-β1 (2 ng/ml), and [3H]thymidine incorporation was determined at various time points. Data represent means of triplicate determinations ± S.E. Exogenous TGF-β1 stimulation of TGF-β1 signals by virtue of competition for TGF-βR-I signaling for inhibition of cell proliferation, activate extracellular matrix synthesis, or activate transcription from a promoter containing TGF-β-responsive elements. Moreover, mutant TGF-βR-II with kinase domain deleted was shown to confer resistance to TGF-β control of developmentally regulated cardiac genes (21). In this study, essentially the same construct was utilized, and stably expressed in cultured renal glomerular capillary endothelial cells. Expression of TGF-βR-IIM mRNA in transfected glomerular capillary endothelial cells was demonstrated by reverse transcriptase-PCR, RNase protection assay, and Northern blot analysis. Although TGF-βR-II is thought to be essential for the propagation of TGF-β1 signals (16). Mutant TGF-βR-II lacking the cytoplasmic signaling domain can be predicted to inhibit TGF-βR-II dependent signals by virtue of competition for TGF-βR-I binding, as long as the ability to heterodimerize is conserved. Chen et al. (19) previously showed that mutant TGF-βR-II lacking the cytoplasmic kinase domain overexpressed in a dominant negative fashion selectively blocked TGF-βR-II signaling for inhibition of cell proliferation. Wieser et al. (20) demonstrated that truncated TGF-βR-II lacking the cytoplasmic domain was able to bind TGF-β1, and form a complex with TGF-βR-I, but failed to inhibit cell proliferation.

**Fig. 4. Effect of TGF-β1 on [3H]thymidine incorporation.** A, cells were incubated in the presence or absence of TGF-β1 (2 ng/ml) for 45 h, followed by [3H]thymidine incorporation into the cells for 3 h. Lane 1, TGF-β1 inhibited [3H]thymidine incorporation in wild-type glomerular endothelial cells to 63 ± 10%. Lane 2, in transfected glomerular endothelial cells carrying TGF-βRIIM, TGF-β1 stimulated [3H]thymidine incorporation to 131 ± 9% (*, p < 0.005, Student’s t test, n = 4). B, transfected glomerular endothelial cells were incubated in 0.5% FBS and in the presence (●) or absence (○) of TGF-β1 (2 ng/ml), and [3H]thymidine incorporation was determined at various time points. Data represent means of triplicate determinations ± S.E. (p < 0.005, analysis of variance). A second experiment gave essentially the same results.

The time course of [3H]thymidine incorporation in transfected cells grown in 0.5% FBS and in the presence or absence of TGF-β1 (2 ng/ml) is shown in Fig. 4B. The results are mean values of triplicate determinations ± S.E. Exogenous TGF-β1-stimulated [3H]thymidine incorporation significantly above the basal rate in 0.5% FBS at 36 and 48 h (p < 0.005, analysis of variance).

Effect of Serum Deprivation on TGF-β1. In cultured wild-type glomerular endothelial cells, with serum deprivation in the presence or absence of dexamethasone pretreatment, many of the cells detached from their substrate while remaining cells organized into capillary-like structures (Fig. 5A). Furthermore, treatment with exogenous TGF-β1, in the presence or absence of dexamethasone, also induced cell detachment and formation of capillary-like structures (Fig. 5B). These events were accelerated by approximately 48 h when compared to those cells under serum deprivation alone. Both cell detachment and formation of capillary-like structures were observed with serum deprivation in mock transfected cells carrying empty vector. In contrast, cell detachment and formation of capillary-like structures were uniformly and entirely absent in transfected cells carrying TGF-βRIIM treated either with serum deprivation (Fig. 5C) or exogenous TGF-β1 (Fig. 5D). When examined with a high power phase-contrast objective, after 5 days of culture the cellular cords formed by wild-type glomerular endothelial cells appeared as tubes that contained a central translucent lumen-like space along their length (Fig. 6A), similar to the in vitro angiogenesis models described by Ingber and Folkman (23) and Montesano et al. (11). Lumen formation was confirmed by transmission electron microscopy, which revealed groups of endothelial cells that were joined by interdigitated cell processes and enclosed a central luminal space, as shown in Fig. 6B. Amorphous material within the lumen, also previously described by Ingber and Folkman (23), likely represents matrix and debris. Clathrin-coated pits and vesicles, as well as cell junctional complex are also observed.

Induction of Apoptosis by Serum Deprivation and Exogenous TGF-β1. Fig. 7 shows genomic DNA size analysis. In wild-type glomerular endothelial cells treated either with serum deprivation or exogenous TGF-β1, DNA fragmentation was observed. This occurred both with and without dexamethasone pretreatment. Genomic DNA fragmentation was absent in transfected glomerular endothelial cells carrying TGF-βRIIM.

Inhibition of Capillary Morphogenesis by Anti-TGF-β1 Antibody. Fig. 8 shows serum-deprived wild-type glomerular endothelial cells, incubated in the absence (panel A) or presence (panel B) of neutralizing antibody to TGF-β1. Cell detachment and formation of capillary-like structures were observed with serum deprivation alone. However, with the addition of neutralizing antibody to TGF-β1, both cell detachment and formation of capillary-like structures were not observed. Additionally, neutralizing antibody to TGF-β1 inhibited DNA fragmentation in serum-deprived wild-type glomerular capillary endothelial cells (data not shown).

**DISCUSSION**

This study sought to explore the role of TGF-β receptors in capillary morphogenesis, using renal glomerular capillary endothelial cells stably transfected with a TGF-βRI construct designed to inhibit TGF-βRIII dependent signals through a transdominant negative action. TGF-β receptors types I and II are co-expressed by most cells (24), and heterodimerize upon ligand binding (16, 17). Heterodimer formation was recently shown to induce phosphorylation of TGF-βRI at the GS domain, an effect dependent on TGF-βRII kinase activity. Phosphorylation of TGF-βRI by TGF-βRII is thought to be essential for the propagation of TGF-β1 signals (16). Mutant TGF-βRII lacking the cytoplasmic signaling domain can be predicted to inhibit TGF-βRI dependent signals by virtue of competition for TGF-βRI binding, as long as the ability to heterodimerize is conserved. Chen et al. (19) previously showed that mutant TGF-βRII lacking the cytoplasmic kinase domain overexpressed in a dominant negative fashion selectively blocked TGF-βRII signaling for inhibition of cell proliferation. Wieser et al. (20) demonstrated that truncated TGF-βRII lacking the cytoplasmic domain was able to bind TGF-β1, and form a complex with TGF-βR-I, but failed to inhibit cell proliferation, activate extracellular matrix synthesis, or activate transcription from a promoter containing TGF-β-responsive elements. Moreover, mutant TGF-βRII with kinase domain deleted was shown to confer resistance to TGF-β control of developmentally regulated cardiac genes (21). In this study, essentially the same construct was utilized, and stably expressed in cultured renal glomerular capillary endothelial cells. Expression of TGF-βRIIIM mRNA in transfected glomerular capillary endothelial cells was demonstrated by reverse transcriptase-PCR, RNase protection assay, and Northern blot analysis. Although TGF-β1 Induces Capillary Morphogenesis and Apoptosis
the \( \alpha \)R-II\(_M\) construct was under a glucocorticoid-regulated promoter, expression was observed even in uninduced conditions, although at lower levels than that in the presence of dexamethasone. That such promoters "leak" during uninduced conditions has previously been observed by others (25).

To demonstrate cell-surface expression and ligand-binding by the mutant receptor, intact cells were incubated with \(^{125}\text{I})\)-labeled proteins by SDS-polyacrylamide gel electrophoresis. In untransfected or mock transfected cells, only the wild-type \( \alpha \)R-I and \( \alpha \)R-II, approximately 70 and 89 kDa, respectively, were observed. In transfected cells, two additional bands of 48 and 36 kDa in size were also observed. Mutant receptor has a predicted molecular mass of 23 kDa. The 48-kDa band is interpreted to represent the mutant receptor with \( \alpha \)GF-\( \beta \)-I dimer bound to it, the 36-kDa band could represent the same mutant receptor with \( \alpha \)GF-\( \beta \)-I monomer or possibly a degradation product of the same receptor. These data are interpreted to show that the mutant receptor is expressed at the cell surface and that it can bind \( \alpha \)GF-\( \beta \).-I. Cross-linking to wild-type \( \alpha \)R-I was less in the transfected cells carrying \( \alpha \)R-II\(_M\) (Fig. 3), when compared to untransfected or mock transfected cells. A plausible explanation is that the mutant receptor competes and heterodimerizes with wild-type \( \alpha \)R-I and is then quickly degraded, internalized, or secreted. Alternatively, the mutant \( \alpha \)R-II could interfere with \( \alpha \)GF-\( \beta \) binding to \( \alpha \)R-I. Inhibition of binding to wild-type \( \alpha \)R-II was not observed under these conditions. This is not unexpected since wild-type \( \alpha \)R-II can bind \( \alpha \)GF-\( \beta \) in the absence of dimerization with \( \alpha \)R-I (16, 17).

Previous in vitro studies have demonstrated that \( \alpha \)GF-\( \beta \) inhibits proliferation of many cell types (26, 27), including endothelial cells (28). In wild-type or mock transfected glomerular endothelial cells, \( \alpha \)GF-\( \beta \) (2 ng/ml) significantly inhibited \(^{3}\text{H}\) thymidine incorporation, whereas in the transfected endothelial cells carrying \( \alpha \)R-II\(_M\), \( \alpha \)GF-\( \beta \) stimulated \(^{3}\text{H}\) thymidine incorporation. This stimulation of \(^{3}\text{H}\) thymidine incorporation by \( \alpha \)GF-\( \beta \) in \( \alpha \)R-II\(_M\) transfected endothelial cells could reflect an alternate receptor signaling pathway such as a \( \alpha \)R-I- mediated response, not dependent on phosphorylation of \( \alpha \)R-I by \( \alpha \)R-II. Time course experiments revealed that in transfected cells, exogenous \( \alpha \)GF-\( \beta \) stimulated \(^{3}\text{H}\) thymidine incorporation significantly above the basal rate in 0.5% FBS at 36 and 48 h (Fig. 4B). In comparison, 5% FBS maximally stimulated \(^{3}\text{H}\) thymidine incorporation between 12 and 24 h (data not shown). These findings suggest that the stimulation may be a secondary rather than a direct mitogenic effect. Our results of stimulation of DNA synthesis are consistent with previous observations that when cells are released from the negative growth regulatory control of \( \alpha \)GF-\( \beta \), cell proliferation occurs and the potential for tumorigenesis can emerge (27, 29).

\( \alpha \)GF-\( \beta \) inhibits proliferation by arresting cells in the G\(_1\) phase and thus interrupting progression through the cell cycle. Laiho et al. (30) observed that \( \alpha \)GF-\( \beta \) prevented phosphorylation of the retinoblastoma gene product and arrested cells in late G\(_1\) phase of the cell cycle. The underphosphorylated retinoblastoma gene product has growth-suppressive function. When progression through the cell cycle is prevented, cells may remain quiescent or withdraw from the cell cycle and undergo terminal differentiation. Indeed, Zentella et al. (31) showed that \( \alpha \)GF-\( \beta \) inhibited cell cycle progression of skeletal myoblasts through the G\(_1\) phase and induced terminal differentiation.

In addition to the inhibition of \(^{3}\text{H}\) thymidine incorporation in wild-type glomerular endothelial cells, cell detachment was observed. Cells that remained on the plate tended to organize into capillary-like structures, a process previously described by others (22, 32, 33). A possible explanation for the observed decreased \(^{3}\text{H}\) thymidine incorporation and cell detachment may be cell cycle arrest and withdrawal, and entry into a
suicide program, or apoptosis. In support of this, proto-oncogene c-myc, an important positive regulator of cell growth induced during the G0/G1 phase of cell cycle, can induce apoptosis under conditions of growth arrest, such as presence of a negative growth regulator, TGF-β1 (34).

Indeed, TGF-β1 has been shown to inhibit cell proliferation and induce apoptosis in rat hepatocytes in vivo (35) and in rabbit uterine epithelial cells in vitro (36). We observed in wild-type glomerular endothelial cells, serum deprivation induced apoptosis as shown by genomic DNA fragmentation and associated capillary formation. Exogenous TGF-β1 treatment accelerated these events. In contrast, genomic DNA fragmentation and associated capillary formation were not observed in transfected endothelial cells expressing TβR-IlM, either with serum deprivation or exogenous TGF-β1 treatment. Since serum deprivation acted as if exogenous TGF-β1 had been added, and since effects of serum deprivation were not seen in cells carrying the transdominant negative mutant receptor, it is plausible that endogenous TGF-β1 might mediate capillary morphogenesis with serum deprivation. In support of this hypothesis, we observed that neutralizing antibody to TGF-β1 abolished both cell detachment and capillary-like tube formation in serum deprived wild-type endothelial cells as well as DNA fragmentation.

In the process of capillary morphogenesis, our studies show that endothelial cells, in response to TGF-β1, undergo a programmed cell death and detach from the substratum, a phenomenon called anoikis (37). The term anoikis is derived from the Greek word for homelessness. Anoikis implies that once cells lose contact with underlying matrix, they undergo programmed cell death, thus preventing these detached cells from establishing themselves in another location. Thus, the phenomenon of anoikis is a mechanism for homeostasis that maintains a certain correct cell number in the body by balancing cell production with cell death. Tumor cells escape this regulation by blocking the apoptotic response. Anoikis may also be important in cell positioning. For instance, in the normal maturation of the skin, the cells that are in contact with the basement membrane proliferate and the cells that migrate away from it into the more superficial layers undergo apoptosis (38). Since integrins are primarily responsible for cell adhesion to ECM, integrin-mediated signaling has been implicated in controlling apoptosis. Frisch and Francis (37) demonstrated that apoptosis was induced by disruption of the interactions between normal epithelial cells and ECM. In endothelial cells, Meredith et al. (39) showed that cells incubated in suspension and denied interactions with ECM rapidly underwent apoptosis. Furthermore, when endothelial cells were plated on an integrin β3 monoclonal antibody, apoptosis was suppressed. Therefore, regulation of apoptosis may be mediated by disruption of cell-matrix interactions and altered cell-cell interactions.

Given this body of evidence, it is not unreasonable to propose that our findings of cell detachment and apoptosis may reflect TGF-β1-mediated matrix degradation. TGF-β1 induces endothelial cell secretion of PA, which is an enzyme that...
deaves the proenzyme plasminogen to form active plasmin. Plasmin is a protease which degrades ECM proteins. Moreover, plasmin activates latent TGF-β1 (12), and thus providing a mechanism for autoamplification loop. ECM proteolysis by proteases such as plasmin facilitates endothelial cell migration and angiogenesis in vivo (11). However, even though apoptosis may be mediated by PA-induced matrix degradation by TGF-β1, this may not be the sole mechanism, in view of the fact that mitogenic growth factors which can prevent apoptosis, such as bFGF, also induces PA.

Angiogenesis is regulated by a number of cytokines in vivo, including bFGF (40), vascular endothelial growth factor (41), and TGF-β1 (6, 7). In our studies, we were able to isolate and delineate the effects of TGF-β1 in vitro from other angiogenic growth factors. Our findings raise the intriguing possibility that apoptosis is a phenomenon necessary in the process of capillary morphogenesis and that both are dependent on TGF-β receptor signaling.

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