Serum-stimulated α₁ type IV collagen gene transcription is mediated by TGF-β and inhibited by estradiol

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Lei, Jun, Sharon Silbiger, Fuad N. Ziyadeh, and Joel Neugarten. Serum-stimulated α₁ type IV collagen gene transcription is mediated by TGF-β and inhibited by estradiol. Am. J. Physiol. 274 (Renal Physiol. 43): F252–F258, 1997.—We examined the hypothesis that fetal calf serum (FCS) stimulates murine mesangial cell α₁ type IV collagen (COL4A1) gene transcription by increasing autocrine production of transforming growth factor-β (TGF-β) through a platelet-derived growth factor (PDGF)-dependent mechanism. PDGF-stimulated COL4A1 gene transcription was inhibited by neutralizing antibody to TGF-β (119.3 ± 3.6 vs. 106.0 ± 6.2 relative luciferase units, expressed as a percentage of control untreated cells, P < 0.003). FCS-stimulated gene transcription was inhibited by neutralizing antibody to PDGF (148.3 ± 4.1 vs. 136.7 ± 0.3 relative luciferase units, P < 0.002) and by neutralizing antibody to TGF-β (148.3 ± 4.1 vs. 127.1 ± 3.4 relative luciferase units, P < 0.036). The inhibitory effect of combined treatment with anti-PDGF and anti-TGF-β antibody on gene transcription was no greater than that of anti-TGF-β antibody alone (129.5 ± 0.53 vs. 127.1 ± 3.4 relative luciferase units, P = ns). FCS-stimulated gene transcription was also inhibited by estradiol (10−7 M) (148.4 ± 3.1 vs. 119.4 ± 8.1 relative luciferase units, P < 0.019). In the presence of estradiol, anti-TGF-β antibody failed to further reduce serum-stimulated gene transcription (119.4 ± 8.1 vs. 115.6 ± 9.8, P = ns), suggesting that estradiol reverses FCS-stimulated COL4A1 gene transcription by antagonizing the actions of TGF-β. Measurement of type IV collagen synthesis by Western blotting confirmed that the intact gene responded in a manner analogous to the promoter construct. These data suggest that FCS stimulates murine mesangial COL4A1 gene transcription, in part, by increasing cellular production of TGF-β and that the ability of estradiol to suppress α₁ type IV collagen synthesis by mesangial cells grown in serum-supplemented media may be mediated by antagonism of the actions of TGF-β.

In the present study, we examined interactions between serum, PDGF, TGF-β, estradiol, and COL4A1 gene transcription in murine mesangial cells. We found that exogenous PDGF-stimulated gene transcription was inhibited by neutralizing antibody to either PDGF or TGF-β. Similarly, FCS-stimulated gene transcription was inhibited by neutralizing antibody to PDGF or to TGF-b. The inhibitory effect of combined treatment with anti-PDGF and anti-TGF-β antibodies on FCS-stimulated COL4A1 gene transcription was no greater than that of anti-TGF-β antibody alone. Estradiol also reversed FCS-stimulated collagen gene transcription. The suppressive effects of estradiol and of anti-TGF-β antibody on FCS-stimulated COL4A1 gene transcription were not additive. Measurement of type IV collagen synthesis by Western blotting confirmed that the intact gene responded in a manner analogous to the promoter construct. These data suggest that FCS stimulates murine mesangial COL4A1 gene transcription, in part, by increasing cellular production of TGF-β and that estradiol suppresses type IV collagen gene transcription by cells grown in serum-supplemented media by antagonizing the effects of TGF-β.

METHODS

Isolation and characterization of murine mesangial cells. Mesangial cells were isolated from kidneys of 8- to 10-wk-old naive SJL/J (H-2b) mice by differential glomerular sieving (33). The present studies were performed on an immortalized, differentiated mesangial cell line transformed with nonreplicating, non-capsid-forming SV40 virus (strain Rh 911). The cells express receptors for angiotensin II and stain positive for Thy-1 antigen, desmin, vimentin, and types I and IV collagens but fail to bind antibody directed against a proximal tubular antigen (41).

Construction of reporter gene plasmids. A plasmid containing portions of the gene encoding murine α1(IV) collagen was linked to luciferase coding sequences to form a reporter construct HB35, as previously described (13). A 2.3-kb XhoI fragment derived from p184 (gift of Dr. P. Killen, Univ. of Michigan) (7, 19) spanning the first three exons, the first two introns and portion of the third intron of the α2(IV) collagen gene, the intergenic bidirectional promoter region, and a portion of the α3(IV) collagen transcription unit was cloned...
into pBluescript (Stratagene, La Jolla, CA) and was truncated within the third intron of the \( \alpha_3 \) (IV) gene by excising the appropriate fragment with Hind III. The fragment was then ligated into the Hind III site of the luciferase expression plasmid, pSVOAlucD5 (10), and its orientation was determined by restriction mapping (22). An additional 3.2-kb fragment of the first intron of the \( \alpha_3 \) (IV) gene, previously shown to modulate the activity of the \( \alpha_3 \) (IV) promoter (7, 19), corresponding to a BamH I-linked 3.2-kb Xba I fragment of the first intron of \( \alpha_3 \) (IV), was inserted into the BamH I site downstream from the 3' end of the luciferase coding sequences, following partial BamH I digestion (22). Intron fragment orientation and position were confirmed by restriction mapping (22). A figure of the reporter gene construct has been previously published (13, 22).

Stable transfection. Murine mesangial cells were cotransfected with HB35 and a selection plasmid pS2V-Neo encoding for neomycin resistance (36) at a molar ratio of 10:1, using the CaPO4-DNA precipitation procedure (3). Cells were then grown in phenol-free, serum-free RPMI 1640 media. FCS increased COL4A1 gene transcription to a level no greater than that obtained with control media (serum-free, no added agents). Cells surviving in medium with Geneticin were expanded and grown in selection medium containing Geneticin (G-418).

Cells were plated in six-well plates and grown in phenol-free, serum-free RPMI 1640. Cells were exposed to varying concentrations of FCS (0, 5, 10, or 20% FCS), recombinant human PDGF (10 ng/ml) (Upstate Biotechnology, Lake Placid, NY), or TGF-\( \beta \) (2 ng/ml) (R & D Systems, Minneapolis, MN) in the presence and absence of rabbit anti-human PDGF neutralizing antibody (20 \( \mu \)g/ml) (R & D Systems), rabbit anti-human TGF-\( \beta \) neutralizing antibody (20 \( \mu \)g/ml) (Upstate Biotechnology), or normal rabbit immunoglobulin G (IgG, 20 \( \mu \)g/ml). In other experiments, cells were treated with 20% FCS in the presence or absence of estradiol (10\(^{-7}\) M) \pm \) rabbit anti-human TGF-\( \beta \) neutralizing antibody (20 \( \mu \)g/ml) (R & D Systems). The estradiol concentration in FCS was 25.38 pg/ml (final concentrations of estradiol: 13 pg/ml, 25 pg/ml, and 51.5 pg/ml in 5, 10, and 20% FCS, respectively).

Because FCS may artifactualy increase cellular protein determinations by contaminating the cell monolayer with serum-derived proteins, cells were extensively washed with phosphate-buffered saline. Cells were then lysed with 100 \( \mu \)l Reporter Lysis Buffer (Promega, Madison, WI) at room temperature for 15 min. Wells were then scraped, and the cell lysate was transferred to a microcentrifuge tube and placed on ice. Tubes were vortexed and microcentrifuged for 2 min at 4°C. The suspension was transferred to a new microcentrifuge tube and stored at \(-70\) °C until assayed. Cell extract (20 \( \mu \)l) was mixed with 10 \( \mu \)l of assay reagent (20 mM tricine, 1.07 mM (Mg\(_2\))\(_2\)Mg(OH)\(_2\), 50 mM Tris, 3% glycerol, 50 mM luciferin, and 350 \( \mu \)M ATP, pH 7.8) at room temperature (all reagents were from Sigma Chemical, St. Louis, MO). Light emission was measured directly at room temperature over a 10-s period in a luminometer (Promega). Blank reactions were determined with equivalent volumes of lysis buffer substituted for cell lysates, and these values were subtracted from experimental values. Luciferase activity was expressed per milligram of cellular protein in the supernatant as relative luciferase units, calculated as the percentage of control untreated cells; \( P < 0.001, n = 13 \) (Fig. 1). FCS (20%) also significantly increased total cellular protein (784 ± 61 vs. 477 ± 35 \( \mu \)g/well, \( P < 0.01 \)). Estradiol reduced 20% FCS-stimulated COL4A1 gene transcription to a level no greater than that obtained in serum-free media (119.4 ± 8.1 (\( n = 5 \)), \( P = NS \), vs. 0% FCS) but failed to reverse the stimulatory effect of FCS.
effect of 20% FCS on total cellular protein (736 ± 89 vs. 784 ± 61 µg/well, *P < 0.003 vs. 0% FCS).

PDGF (10 ng/ml) stimulated COL4A1 gene transcription by mesangial cells grown in the absence of serum (119.3 ± 3.6 relative luciferase units; *P < 0.001 vs. control) (Fig. 2). PDGF-stimulated reporter gene transcription was reversed by neutralizing antibody to PDGF (119.3 ± 3.6 vs. 99.1 ± 2.1 relative luciferase units; *P < 0.001, n = 3). Antibody alone and normal rabbit IgG had no effect on reporter gene transcription in cells grown in the absence of serum (100.0 ± 1.1, 104.6 ± 3.7, and 96.3 ± 2.1 relative luciferase units, respectively; *P = NS vs. 0% FCS).

TGF-β1 (2 ng/ml) stimulated COL4A1 gene transcription by mesangial cells grown in serum-free media (156.0 ± 11.3 relative luciferase units; *P < 0.001 vs. control, n = 5). Anti-TGF-β neutralizing antibody reversed TGF-β-stimulated reporter gene transcription (156.0 ± 11.3 vs. 106.6 ± 3.9 luciferase units; *P < 0.001, n = 5). Antibody alone and normal rabbit IgG had no effect on reporter gene transcription in cells grown in the absence of serum (104.6 ± 3.7 and 96.3 ± 2.1, *P = NS vs. 0% FCS).

Anti-TGF-β neutralizing antibody inhibited the stimulatory effects of 20% FCS on reporter gene transcription by 37% (148.3 ± 4.1 (n = 9) vs. 127.1 ± 3.4 (n = 8) relative luciferase units; *P < 0.005). Anti-TGF-β antibody had no significant effect on the increase in total cellular protein induced by FCS (854 ± 84 vs. 784 ± 61 µg/well, *P = NS). Similarly, anti-PDGF neutralizing antibody inhibited the stimulatory effects of 20% FCS on reporter gene transcription (148.3 ± 4.1 (n = 9) vs. 136.7 ± 0.3 (n = 3) relative luciferase units, *P < 0.002) (Fig. 3). However, anti-PDGF antibody had no significant effect on the increase in total cellular protein induced by 20% FCS (872 ± 49 vs. 784 ± 61 µg/well, *P = NS). The inhibitory effect of combined treatment with anti-PDGF and anti-TGF-β antibodies on collagen IV gene transcription was no greater than that of anti-TGF-β antibody alone (129.5 ± 0.5 (n = 3) vs. 119.3 ± 3.6 relative luciferase units; *P < 0.001 vs. control).

The inhibitory effect of combined treatment with anti-PDGF and anti-TGF-β antibodies on collagen IV gene transcription was no greater than that of anti-TGF-β antibody alone (129.5 ± 0.5 (n = 3) vs. 119.3 ± 3.6 relative luciferase units; *P < 0.001 vs. control).

Fig. 1. Effects of fetal calf serum (FCS) and estradiol on type IV collagen gene transcription as assessed by activity of a collagen IV/luciferase gene construct. FCS stimulated α1 type IV collagen gene transcription in a dose-dependent manner. FCS-stimulated gene transcription was reversed by estradiol (10−7 M). Solid bars, fetal calf serum; hatched bars, fetal calf serum + estradiol. *P < 0.001 vs. 0% FCS.
Fig. 4. Effects of anti-TGF-β neutralizing antibody and of estradiol on 20% FCS-stimulated collagen IV gene transcription. FCS-stimulated gene transcription was inhibited by either estradiol (10⁻⁷ M) or anti-TGF-β antibody (20 µg/ml). Effects of estradiol and anti-TGF-β antibody were not additive. *P < 0.001 vs. 0% FCS, †P < 0.019 vs. 20% FCS. ‡P < 0.005 vs. 20% FCS. §P < 0.005 vs. 20% FCS.

vs. 127.1 ± 3.4 (n = 8) relative luciferase units, P = NS. Normal rabbit IgG had no effect on 20% FCS-stimulated COL4A1 gene transcription (159.2 ± 7.4 (n = 4) vs. 148.3 ± 4.1 (n = 9), P = NS) or on total cellular protein (736 ± 89 vs. 784 ± 61 µg/well, P = NS).

Estradiol inhibited the stimulatory effect of 20% FCS on COL4A1 gene transcription [148.4 ± 3.1 (n = 13) vs. 119.4 ± 8.1 (n = 5) relative luciferase units, P < 0.019] (Fig. 4). In the presence of estradiol, anti-TGF-β antibody failed to further reduce COL4A1 gene transcription [119.4 ± 8.1 (n = 5) vs. 115.6 ± 9.8 (n = 3) relative luciferase units, P = NS] (Fig. 4).

We measured type IV collagen protein by Western blotting to confirm that intact cells respond to serum and other agents in a manner analogous to the reporter gene construct. As shown in Fig. 5, type IV collagen protein was increased in 20% FCS-treated cells (323.8 ± 24.6%, expressed as % of control values, P < 0.001 vs. control). This increase was reversed by anti-TGF-β antibody (128.8 ± 13.2%, P < 0.01 vs. 20% FCS) and by anti-PDGF antibody (132.6 ± 15.7%, P < 0.01 vs. 20% FCS). The rise in type IV collagen protein was also inhibited by estradiol (144.6 ± 20.4%, P < 0.01 vs. 20% FCS). Neither anti-TGF-β antibody, anti-PDGF antibody, nor estradiol had any effect on type IV collagen protein in control cells.

DISCUSSION

The beneficial effect of female gender on the development and progression of atherosclerosis may reflect, in part, reduced accumulation of vascular wall extracellular matrix (35). In this regard, estrogen administration reduces collagen deposition in the aorta of hypertensive and hypercholesterolemic animals and reduces type I and type III collagen synthesis by vascular smooth muscle cells in vitro (28). Analogous to its effects on atherosclerosis, female gender has been associated with a slower rate of progression of renal disease (35). The accumulation of glomerular extracellular matrix after renal injury is a precursor to the development of glomerular obsolence and progressive loss of renal function (23). The ability of estradiol to suppress type IV collagen synthesis by mesangial cells grown in serum-supplemented media may translate into reduced accumulation of collagen after glomerular injury in females (23).

FCS contains numerous growth factors, including PDGF and small quantities of TGF-β (18, 26, 38). In addition, FCS stimulates mesangial cells to increase TGF-β mRNA in a dose-dependent manner (18). This latter effect may be mediated by PDGF present in the serum (1, 2). It has been suggested that the stimulatory effect of FCS on the synthesis of several mesangial matrix components is mediated by the ability of PDGF present in serum to stimulate cellular synthesis of TGF-β and initiate autocrine effects (21).

Numerous studies demonstrate autocrine and paracrine interactions between PDGF and TGF-β. Recombinant human PDGF increases the steady-state level of TGF-β mRNA and induces the release of TGF-β protein from cultured human mesangial cells (2, 4). PDGF also increases TGF-β mRNA and/or protein in rat kidney fibroblasts, murine macrophages, human articular chondrocytes, and human renal proximal tubular cells (20, 31, 39, 40). Consistent with the observation that PDGF stimulates TGF-β synthesis, glucose-stimulated TGF-β synthesis in human mesangial cells and renal proximal tubular cells is mediated through release of PDGF (11, 30). Anti-PDGF-β antibody reverses the increase in TGF-β gene expression and protein synthesis induced by high glucose (11, 30). Also consistent with the observation that PDGF upregulates mesangial cell TGF-β expression, the effects of PDGF on mesangial cell matrix protein synthesis are reversed by anti-TGF-β antibody (27, 38). In contrast, a single study failed to find an effect of exogenous PDGF on TGF-β mRNA levels in rat mesangial cells (18).
Addition of PDGF to human mesangial cells increases fibronectin and type III collagen synthesis by stimulating the intermediate production of TGF-$\beta$ (27, 38). The PDGF-stimulated increase in each of these matrix proteins is inhibited by the addition of a neutralizing antibody to TGF-$\beta$ (27, 38). Mesangial cells grown in media containing a high glucose concentration or exposed to advanced glycosylation end products show enhanced type III collagen production which is mediated, in part, by increased cellular production of PDGF (38). Most of the stimulatory effect of PDGF on type III collagen synthesis under these conditions is due to the intermediate production of TGF-$\beta$ (38). High glucose also induces a biphasic growth response in mesangial cells, with early growth stimulation mediated by PDGF and later growth inhibition mediated by PDGF induction of TGF-$\beta$ (11). In contrast to these observations, a single study failed to find a significant effect of anti-PDGF antibody on collagen IV mRNA in mesangial cells grown in 20% FCS (12).

In most studies, TGF-$\beta$ has been shown to increase the abundance of collagenase-sensitive protein and to stimulate types I and IV collagen synthesis in rat, murine, and human glomerular mesangial cells (8, 14, 25, 37, 42). This stimulation occurs at the transcriptional level, as reflected in an increase in mRNA levels in the absence of any increase in mRNA transcript stability (5, 14, 17, 37). In addition to its effect on collagen synthesis in rat and murine mesangial cells, TGF-$\beta$ induces cellular hypertrophy and increases total cellular protein normalized for cell number (5, 8, 25).

We have previously shown that estradiol reverses the stimulatory effect of TGF-$\beta$ on mesangial cell COL4A1 gene transcription, using a minigene construct (34). This observation, in addition to those summarized above, led us to hypothesize that serum stimulates mesangial cells to increase their synthesis of $\alpha_1$-type IV collagen by increasing cellular production of TGF-$\beta$ and that the ability of estradiol to suppress the synthesis of $\alpha_1$-type IV collagen by mesangial cells grown in serum-supplemented media may be mediated via antagonism of the actions of TGF-$\beta$.

In the present study, we examined interactions between serum, PDGF, TGF-$\beta$ and estradiol in COL4A1 gene transcription in murine mesangial cells. We found that PDGF-stimulated collagen IV gene transcription is inhibited by neutralizing antibody to TGF-$\beta$, suggesting that increased cellular production of TGF-$\beta$ mediates, in part, the stimulatory effects of PDGF on COL4A1 gene transcription. We also found that FCS stimulated COL4A1 gene transcription in a dose-dependent manner. FCS-stimulated gene transcription was inhibited by neutralizing antibody to either PDGF or TGF-$\beta$. The observation that the inhibitory effect of combined treatment with anti-PDGF and anti-TGF-$\beta$ antibodies on COL4A1 gene transcription were no greater than that of anti-TGF-$\beta$ antibody alone implies that these cytokines act through a common mechanism. Our data show that more than one-third of the increase in COL4A1 gene transcription induced by 20% FCS was mediated by PDGF-stimulated TGF-$\beta$ production. In contrast, serum-induced increases in total cellular protein content were not suppressed by anti-TGF-$\beta$ or anti-PDGF neutralizing antibody.

A 3.2-kb sequence in the first intron of the $\alpha_1$ IV gene modulates the activity of the collagen IV promoter (29, 32). This sequence contains a motif with close homology to a serum response element found in the gene encoding the human 70-kDa heat shock protein (6). Nuclear extracts from Engelbreth-Holm-Swarm tumor cells or differentiated F9 cells show DNA footprints that cover the region containing the serum response element (6). Thus serum-induced binding of transcription factors to this motif may be responsible for the component of serum-stimulated COL4A1 gene transcription that is TGF-$\beta$ independent.

Although estradiol had no effect on COL4A1 gene transcription in cells grown in the absence of serum, estradiol suppressed FCS-stimulated gene transcription. Of note, the TGF-$\beta$-mediated component of serum-stimulated collagen IV gene transcription is of sufficient magnitude to account for the suppressive effects of estradiol observed in serum-supplemented media. In the presence of estradiol, anti-TGF-$\beta$ antibody failed to further reduce FCS-stimulated COL4A1 gene transcription. These data suggest that FCS stimulates murine mesangial cell type IV collagen gene transcription, in part, by increasing cellular production of TGF-$\beta$ and that estradiol suppresses type IV collagen gene transcription by cells grown in serum-supplemented media by antagonizing the effects of TGF-$\beta$.

The increase in type IV collagen protein observed in cells treated with 20% FCS exceeded the magnitude of stimulation of COL4A1 gene transcription induced by FCS. This discrepancy may reflect additional posttranscriptional events.

We have only studied transcription of the $\alpha_1$ chain of type IV collagen. However, the peculiar arrangement of the $\alpha_1$ and $\alpha_2$ chains of type IV collagen (head-to-head on the same chromosome, sharing a common bidirectional promoter) implies some common control mechanism for both genes (7). Moreover, since both chains form a triple helix with two $\alpha_1$ chains and one $\alpha_2$ chain, there usually exists concordance of transcriptional regulation in a ratio of 2:1 (6).

The murine $\alpha_1$ and $\alpha_2$ type IV collagen genes are located on chromosome 13 in a head-to-head orientation separated by a common bidirectional promoter region spanning 130 base pairs (7). Kuncio et al. (22) have recently shown that the common promoter is sufficient to confer positive regulation by TGF-$\beta_1$ in murine renal tubular cells. The common promoter contains binding sites for the transcription factor Sp1 (GGGCGG) but lacks an estrogen responsive element (7). Sp1 has been shown to mediate the effects of TGF-$\beta$ on the transcription of a number of genes, including the genes for $\alpha_1$(I) and $\alpha_2$(I) collagen and the cyclin-dependent kinase inhibitors p21 and p15INK4B (9, 15, 16, 24). In preliminary studies, we have shown that nuclear extracts from mesangial cells treated with TGF-$\beta$ show...
increased binding to an Sp1 site in the promoter of the type IV collagen gene and that estradiol reverses this enhanced binding (34). These observations are consistent with the hypothesis that Sp1 is involved in the regulation of collagen IV synthesis by TGF-β and that estradiol reverses the stimulatory effects of TGF-β on collagen IV synthesis via interactions with Sp1.

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Received 18 March 1997; accepted in final form 18 September 1997.

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