Cell Density-dependent Regulation of Proteoglycan Synthesis by Transforming Growth Factor-β1 in Cultured Bovine Aortic Endothelial Cells*

Toshiyuki Kaji‡, Akihiro Yamada, Sawako Miyajima, Chika Yamamoto, Yasuyuki Fujiwara, Thomas N. Wight§, and Michael G. Kinsella§

From the Department of Environmental Health, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan and the Department of Pathology, School of Medicine, University of Washington, Seattle, Washington 98195-7470

The regulation of vascular endothelial cell behavior during angiogenesis and in disease by transforming growth factor-β1 (TGF-β1) is complex, but it clearly involves growth factor-induced changes in extracellular matrix synthesis. Proteoglycans (PGs) synthesized by endothelial cells contribute to the formation of the vascular extracellular matrix and also influence cellular proliferation and migration. Since the effects of TGF-β1 on vascular smooth muscle cell growth are dependent on cell density, it is possible that TGF-β1 also directs different patterns of PG synthesis in endothelial cells at different cell densities. In the present study, dense and sparse cultures of bovine aortic endothelial cells were metabolically labeled with [3H]glucosamine, [35S]sulfate, or [35S]labeled amino acids in the presence of TGF-β1. The labeled PGs were characterized by DEAE-Sepharose ion exchange chromatography and Sepharose CL-4B molecular sieve chromatography. The glycosaminoglycan Mr and composition were analyzed by Sepharose CL-6B chromatography, and the core protein Mr was analyzed by SDS-polyacrylamide gel electrophoresis, before and after digestion with papain, heparitinase, or chondroitin ABC lyase. These experiments indicate that the effect of TGF-β1 on vascular endothelial cell PG synthesis is dependent on cell density. Specifically, TGF-β1 induced an accumulation of small chondroitin/dermatan sulfate PGs (CS/DSPGs) with core proteins of ∼50 kDa in the medium of both dense and sparse cultures, but a cell layer-associated heparan sulfate PG with a core protein size of ∼400 kDa accumulated only in dense cultures. Moreover, only in the dense cell cultures did TGF-β1 cause CS/DSPG hydrodynamic size to increase, which was due to the synthesis of CS/DSPGs with longer glycosaminoglycan chains. The heparan sulfate PG and CS/DSPG core proteins were identified as perlecan and biglycan, respectively, by Western blot analysis. The present data suggest that TGF-β1 promotes the synthesis of both perlecan and biglycan when endothelial cell density is high, whereas only biglycan synthesis is stimulated when the cell density is low. Furthermore, glycosaminoglycan chains are elongated only in biglycan synthesized by the cells at a high cell density.

Proteoglycans (PGs) are macromolecules that are composed of a core protein and one or more glycosaminoglycan (GAG) side chain(s) as a common feature (1). Vascular endothelial proteoglycans are involved in the regulation of arterial properties such as permeability, lipid metabolism, hemostasis, thrombosis, and extracellular matrix assembly (2–5). In addition, PGs can regulate endothelial cell functions that are mediated by growth factors and cytokines, such as transforming growth factor-β (TGF-β) and basic fibroblast growth factor (FGF-2), to which some PGs bind. Endothelial cells synthesize heparan sulfate PGs (HSPGs), including the major endothelial extracellular matrix PG, perlecan (6), members of the syndecan family of transmembrane PGs (7, 8), and the cell surface-associated PG, glypicin-1 (7). Vascular endothelial cells also constitutively synthesize and secrete the large aggregating chondroitin sulfate PG, versican (9), and the small leucine-rich chondroitin/dermatan sulfate PG (CS/DSPG), biglycan (10, 11). In addition, the expression of another small leucine-rich CS/DSPG, decorin, is induced during formation of neovessels both in vitro (12, 13) and in vivo (14).

TGF-β1 is synthesized by endothelial cells and, when activated, dramatically modifies endothelial cell proliferation and migration (15, 16). The cytokine is also released from aggregated platelets after injury of the vascular endothelium (17) and may contribute to the development of the atherosclerotic lesion (18). A hallmark of the atherosclerotic lesion is the accumulation of extracellular matrix components such as PGs and collagen (19–22). While it is well known that TGF-β regulates the synthesis of various PGs in several cell types, including fibroblasts (23–25), epithelial cells, myoblasts (26), and vascular smooth muscle cells (27, 28), whether this cytokine influences the production of specific PGs by endothelial cells is not known.

The cytokine-driven accumulation of extracellular matrix macromolecules in the vessel wall during disease and in other sites of TGF-β release and activation, such as at wound sites, clearly fills a structural function. However, the induced expression of some PGs by TGF-β may also influence cell proliferation, migration, and phenotype by modifying the utilization and activity of growth factors at sites of PG deposition. For example, heparan sulfate chains borne on PGs such as the large basement membrane-associated HSPG, perlecan, promote the binding of FGF-2 to its receptor (29, 30), suggesting that perlecan in endothelial extracellular matrix may regulate...
endothelial cell functions such as proliferation, migration, angiogenesis, and fibrinolytic activity mediated by TGF-β (31, 32). The small leucine-rich PGs biglycan and decorin bind, via their core proteins, to TGF-β and sequester the cytokine from the cell surface receptor (33–35). It is thus likely that the regulation of endothelial cell functions mediated by the cytokines may be influenced by changes in the PGs that are synthesized.

In the present study, PGs synthesized by cultured bovine aortic endothelial cells after exposure to human platelet TGF-β1 were characterized to clarify the regulation of endothelial PG synthesis by TGF-β. Since the growth response of sparse cultures of vascular smooth muscle cells to TGF-β is different from dense cultures of the cells (36), we tested whether TGF-β also regulates PG synthesis by vascular endothelial cells in a cell density-dependent manner. The results suggest that, like the effect of TGF-β on cell proliferation, cells respond differently to the cytokine at different cell densities. This difference in response may have important consequences for the control of endothelial cell behavior during repair of vascular damage.

**EXPERIMENTAL PROCEDURES**

**Materials—**Vascular endothelial cells derived from bovine aorta and Western blotting blocking reagent were from Dainihon Pharmaceutical (Osaka, Japan). RPMI 1640 medium and ASF 301 medium, which contained 10 ng/ml epidermal growth factor, 1 μg/ml insulin, 10 μg/ml transferrin, and 100 μg/ml bovine serum albumin were from Nissui Pharmaceutical (Tokyo, Japan) and Ajinomoto (Tokyo, Japan), respectively. Fetal bovine serum was from Summit Biotechnology (Collinsville, IL). Tissue culture dishes and plates were from Corning (Corning, NY). n-[6-3H]Glucosamine hydrochloride (1480 GBq/mmol), [35S]Na2SO4 (carrier-free), and Enlightening Enhancer were from NEN Life Science Products; 1-[14C]lucine (10.8 GBq/mmol) and Tran-3-S-label metabolic labeling reagent, which consists of 71% L-[14C]methionine, 15% L-[35S]cysteine, and other 35S-labeled compounds, were from ICN Pharmaceuticals (Irvine, CA). TGF-β-neutralizing antibody was from Genzyme (Cambridge, MA). Sepharose CL-4B, Sepharose CL-6B and PD-10 columns (disposable Sephadex G-25M) were from Amersham Pharmacia Biotech; DEAE-Sephasil, benzamidine, Tris base, dextran blue, phenylmethanesulfonyl fluoride, papain (1.7 units/mg solid), and heparin (5.0 mg/ml) were from Provi-Test, Kogyo (Tokyo, Japan). Rabbit antiserum against placentor core protein (EY-9) and biglycan core protein (LF-96) were kindly provided by Dr. John Hassell (Shiriner’s Hospital for Crippled Children, Tampa, FL) and Dr. Larry Fisher (NIDR, National Institutes of Health, Bethesda, MD), respectively. Trypsin (1:250) and Pronase were from Difco and Roche Molecular Biochemicals, respectively; etylypyridinium chloride (CPC), and other reagents were from Nakalai Tesque (Kyoto, Japan).

**Incorporation of [3H]Glucosamine and [35S]Sulfate into Glycosaminoglycans—**Vascular endothelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in 100-mm dishes in a humid atmosphere of 5% CO₂ in air until confluent. They were transferred into 24-well culture plates at 1 × 10⁶ cells/well and cultured for 24 h ("sparse cultures") or until confluent ("dense cultures"). The medium was replaced by fresh serum-free ASF 301 medium after washing of the cells with the medium, and the cells were incubated at 37 °C for 4, 8, 24, or 48 h in 0.25 ml of fresh serum-free ASF 301 medium with TGF-β1 at 0.1, 1, or 10 ng/ml in the presence of both 100 kBq/ml [3H]glucosamine and 370 kBq/ml [35S]sulfate. After incubation, the conditioned medium was harvested, and the cell layer was washed with 0.25 ml of Ca²⁺− and Mg²⁺−free phosphate-buffered saline (CMF-PBS); the wash was combined with the corresponding conditioned medium. The cell layer was incubated at 37 °C for 5 min with 0.25 ml of CMF-PBS containing 0.25% trypsin and 0.02% EDTA. The trypsinized cell suspension was harvested, and the cell culture was washed with 0.25 ml of CMF-PBS. The wash was combined with the corresponding cell suspension and centrifuged at 1500 × g for 5 min. The supernatant included GAGs derived from the cell surface and the solubilized extraacellular matrix (37). The supernatant and the conditioned medium were used for the determination of radioactive GAGs by the CPC precipitation method (38) as follows: each fraction was incubated with 3 mg/ml Pronase at 50 °C for 3 h. The Pronase digests were mixed with 4 mg/ml carrier chondroitin sulfate A and 0.5% CPC. After incubation at 37 °C for 24 h, the mixture was washed at 1500 × g for 10 min to obtain the precipitated GAG-CPC complexes. The precipitate was dissolved in 0.1 ml of 4 M NaCl and reprecipitated by the addition of 1.4 ml of 80% aqueous ethanol. The CPC precipitation, the centrifugation, the NaCl dissolution, and the ethanol precipitation were repeated three times. The precipitate was collected by centrifugation at 1500 × g for 10 min and dissolved in water. The incorporated radioactivity was measured by liquid scintillation counting.

**Cell Number and Protein Synthesis—**Dense and sparse cultures of vascular endothelial cells were incubated at 37 °C for 48 h in 1 ml of serum-free ASF 301 medium with or without TGF-β1, at 0.1, 1, or 10 ng/ml medium for 48 h and labeled with 2.5 kBq/ml [3H]leucine during the last 6 h of the incubation. After incubation, the medium was discarded, and the cells were washed twice with CMF-PBS. The cells were then scraped off with a rubber policeman in the presence of 0.75 ml of CMF-PBS. The culture well was washed with 0.75 ml of CMF-PBS, and the wash was combined with the harvested cell suspension. Cell homogenate was prepared by sonication of the cell suspension, and the incorporation of [3H]leucine into the 5% trichloroacetic acid-insoluble fraction of the cell homogenate was determined by liquid scintillation counting. A portion of the cell homogenate was used for DNA content by the fluorometric method (39), and the incorporated radioactivity was expressed as dpm/μg of DNA. Separately, dense and sparse cultures of endothelial cells were prepared in 24-well plates and treated with TGF-β1 for 48 h. The cells were then harvested by trypsinization, and the cell number at the end of the treatment was counted with a particle counter (Sysmex CDA-500).

**Dissociative Proteoglycan Extraction—**Dense and sparse cultures of vascular endothelial cells were prepared in 100-mm dishes and incubated at 37 °C for 48 h in 6 ml of serum-free ASF 301 medium with TGF-β1 at 10 ng/ml in the presence of 200 kBq/ml [3H]glucosamine. The conditioned medium was harvested, and solid urea was added to a final concentration of 8 M. The cell layer was washed twice with ice-cold CMF-PBS. After extraction with 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM N-ethylmaleimide, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4 °C for 15 min, the layer was harvested by scraping with a rubber policeman. The medium and cell extracts were chromatographed on PD-10 columns equilibrated in 8 M urea, 0.01 M Tris (pH 7.5) containing 2 mM EDTA, 0.1 mM NaCl, 0.5% Triton X-100, and 50 mM Tris base to obtain high molecular mass (>3 kDa) macromolecules.

**Characterization of Proteoglycans—**To separate PGs on the basis of differences in charge density, the macromolecules were applied to DEAE-Sephasil (5 ml of resin) in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.1 mM NaCl, 0.5% Triton X-100, and 50 mM Tris base.

- **Ultraviolet absorbance** was determined from the column with 300 ml of the buffer. Bound radioactivity was eluted from the column with a liner gradient of 0.1–0.7 M NaCl in the urea (total volume of 50 ml). Proteoglycan-containing peaks are eluted at ~0.4 and 0.5 M NaCl (peaks II and III, respectively) and succeed an earlier eluting peak (peak I, ~0.2 M NaCl) that does not contain PGs (40). Peak II in the cell layer of dense cells and peak III in the conditioned medium of either dense or sparse cells were separately pooled because they contained markedly increased radioactivity in samples from TGF-β-treated cultures ("see Results"). Pooled peak material was concentrated by application of the diluted samples to 0.3-mL DEAE-Sephasil columns and eluting bound radioactivity with sequential washes of 8 M urea buffer containing 3 M NaCl. Concentrated PGs were then chromatographed on a Sepharose CL-4B column (0.9 × 80 cm) in 8 M urea buffer containing 0.25 M NaCl to separate PGs on the basis of their hydrodynamic size. The high and low Mᵤ subclasses of peak II and the low Mᵤ subclass of peak III, defined on the basis of Sepharose CL-4B elution position, were pooled and concentrated by DEAE-Sephasil minicolumns.

PGs were precipitated with 3.5 volumes of 1.5% potassium acetate in 95% ethanol and 80 μg/ml carrier chondroitin sulfate for 2 h at 4 °C. The precipitates were repeatedly three times. The precipitated PGs were resuspended with 10 μg of papain in 0.1 mM acetate buffer (pH 7.0) containing 5 mM EDTA and 5 mM cysteine at 65 °C for 4 h or with 17 units/ml chondrin-olute ABC lyase in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mg/ml bovine serum albumin and 3 mM sodium acetate for at 37 °C for 4 h or with 1 unit/ml heparitinase in 10 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM calcium acetate at 37 °C for 4 h. The digested sam-
In dense cultures, the incorporation of both [3H]glucosamine and [35S]sulfate into GAGs was significantly increased by TGF-β1 in both the cell layer and the conditioned medium (Fig. 1). In contrast, TGF-β1 significantly increased [3H]glucosamine and [35S]sulfate incorporation only into GAGs that accumulated in the conditioned medium, but not in the cell layer, of cultures of sparse cells. In compartments of these cultures in which GAG accumulation was increased by TGF-β1, the increased accumulation was significant at 1 ng/ml and further increased at 10 ng/ml, indicating that the regulation of GAG accumulation by TGF-β1 is dose-dependent. A time course of the stimulation of endothelial GAG synthesis by TGF-β1 at 10 ng/ml (Fig. 2) indicated that the increase in [3H]glucosamine incorporation was significant after 8 h in the cell layer and the medium of the dense cultures but was significantly different from unstimulated controls only after 24 h in sparse culture medium. At no time after stimulation with TGF-β1, was [3H]glucosamine incorporation into GAGs increased over control in the cell layer of sparse cell cultures. In summary, sparse and dense cultures differed in response to TGF-β1 stimulation primarily by the accumulation of GAG in the cell layers of dense but not sparse cultures. Since TGF-β1 increased the [3H]glucosamine incorporation in a time-dependent manner, vascular endothelial cells were treated with TGF-β1 at 10 ng/ml for 48 h in the following experiments.

To confirm that the observed increase in endothelial GAG synthesis was dependent directly upon TGF-β1, cells were treated with the cytokine in the presence or absence of TGF-β1 neutralizing antibody, and the incorporation of [3H]glucosamine into GAGs was examined (Fig. 3). The TGF-β1-induced increase in [3H]glucosamine incorporation in the cell layer and the conditioned medium of the dense cells and in the conditioned medium of the sparse cells was completely abrogated in the presence of the antibody, indicating that the increase in the [3H]glucosamine incorporation requires TGF-β1. The antibody alone did not decrease the control levels of [3H]glucosamine incorporation into GAGs, suggesting that endogenous TGF-β1 is not activated in either sparse or dense endothelial cell cultures under the conditions of the experiments.

The observed TGF-β1-induced increase in the accumulation of GAGs in the cell layer and the conditioned medium of vascular endothelial cells may be due to an increase in cell number or represent an increase in general protein synthesis. To address these questions, the cell number and the incorporation of [14C]leucine into acid-insoluble macromolecules after exposure to the cytokine were examined. Over 48 h, the number of sparsely plated endothelial cells was significantly but only slightly decreased by 10 ng/ml TGF-β1, when compared with controls, whereas the number of cells in dense cultures was unaffected by the cytokine (Table 1). Moreover, TGF-β1 did not change overall protein synthesis by either dense or sparse cultures, as assessed by [14C]leucine incorporation into trichloroacetic acid-precipitable radioactivity. These results indicate that the TGF-β1-induced increase in the [3H]glucosamine and [35S]sulfate incorporation into GAGs cannot be explained by increased cell number and does not represent an overall increase in protein synthesis.

The cell layer and the medium extracts of [3H]glucosamine-labeled dense and sparse cultures were submitted to a DEAE-Sepharose column to separate PGs based on charge density differences. In sparse and dense cultures, either with or without TGF-β1 treatment, incorporated [3H]glucosamine radioactivity eluted from the ion exchange column by the NaCl gradient in three peaks, at approximately 0.2, 0.4, and 0.5 M NaCl (Figs. 4 and 5). Previous works (40, 43) have determined that PGs elute in the second and third peaks. TGF-β1 markedly...
increased the radioactivity that elutes in the second peak of the cell layer and that of the third peak of conditioned medium extracts of dense cell cultures (Fig. 4). In comparison, only the radioactivity in the third peak of the conditioned medium extracts was markedly increased by TGF-β1 in cultures of sparse cells (Fig. 5). These results are consistent with the observation of a TGF-β1-induced increase in the accumulation of GAGs in the cell layer and the conditioned medium of dense endothelial cells and only in the conditioned medium of the sparse cells (Figs. 1 and 2).

To identify and characterize newly synthesized PGs, DEAE-Sephacel peaks that were selectively increased by TGF-β1 treatment were pooled as shown (Figs. 4 and 5, bars). DEAE-Sephacel peak II from the cell layer of the dense cultures and peak III from the conditioned medium of both dense and sparse cultures were separated on the basis of their hydrodynamic size by Sepharose CL-4B molecular sieve chromatography as shown in Figs. 6 and 7. Peak II derived from the dense endothelial cell layer was separated into two subclasses: a high Mw subclass (HMW) that eluted near the void volume and a low Mw subclass (LMW) that eluted at a $K_v$ of 0.5–0.9 (Fig. 6). TGF-β1 increased the radioactivity in both subclasses. DEAE-Sephacel peak III derived from conditioned media of either dense (Fig. 6) or sparse (Fig. 7) cultures was also separated into the high and low Mw subclasses. The low Mw subclass was predominant and increased by TGF-β1, in medium extracts of cultures of either cell density. However, the elution position of the low Mw subclass was shifted from $K_v$ of 0.49 to 0.38 in extracts from dense cultures treated with TGF-β1, while the elution position of the low $M_w$ subclass was unaffected by TGF-β1 treatment of sparse cell cultures ($K_v$ values in the control and TGF-β1 were both 0.53). This observation suggests that the hydrodynamic size of the low $M_w$ subclass of peak III was increased by the cytokine only in dense cultures of cells.

Analysis of GAG composition was performed to aid in the identification of the PGs that were present in the peaks isolated by molecular sieve chromatography. Thus, GAG chains were isolated after papain digestion from PGs changed in amount by TGF-β1 treatment (i.e. the high and low $M_w$ subclass of peak II PG from the dense cell layer (Fig. 6, bars) and the low $M_w$ subclass of peak III PGs from the medium of both dense and sparse cultures (bars in Figs. 6 and 7, respectively) and tested for sensitivity to digestion by heparitinase and chondroitin ABC. GAGs from both the high $M_w$ subclass of peak II PG from the dense cell layer (Fig. 6, bars) and the low $M_w$ subclass of peak III PGs from the medium of both dense and sparse cultures were digested with chondroitin ABC lyase. Thus,

### Table I

| Cell number | $[^{14}C]$Leucine |
|------------|-----------------|
| $10^5/cm^2$ | dpm/μg DNA |
| **Dense cultures** | |
| Control | 826 ± 59 | 3747 ± 147 |
| 0.1 ng/ml TGF-β1 | 870 ± 60 | 3921 ± 177 |
| 1.0 ng/ml TGF-β1 | 945 ± 81 | 4355 ± 316 |
| 10 ng/ml TGF-β1 | 881 ± 56 | 4118 ± 118 |
| **Sparse cultures** | |
| Control | 574 ± 25 | 545 ± 79 |
| 0.1 ng/ml TGF-β1 | 494 ± 38 | 740 ± 35 |
| 1.0 ng/ml TGF-β1 | 446 ± 18$a$ | 586 ± 20 |
| 10 ng/ml TGF-β1 | 428 ± 17$a$ | 545 ± 40 |

*$^a$Significantly different from the corresponding control, $p < 0.01$. 

**Fig. 3.** The incorporation of $[^{3}H]$glucosamine into GAGs accumulated in the cell layer and the conditioned medium of dense and sparse vascular endothelial cells after exposure to TGF-β1 in the presence or absence of TGF-β1-neutralizing antibody. Dense and sparse cultures of bovine aortic endothelial cells were incubated at 37 °C for 48 h with TGF-β1 (10 ng/ml) and/or TGF-β1-neutralizing antibody in the presence of $[^{3}H]$glucosamine. Values are means ± S.E. of four samples. Significant difference from the corresponding control is shown as follows. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student’s t test).
However, the effect of TGF-β low on cell density, because the length of GAG chains borne by the CS/DSPGs and HSPGs. As shown in Table II, the low TGF-β treatment altered the GAG chain length of [3H]glucosamine-labeled PGs extracted from the cell layer and the conditioned medium of sparse vascular endothelial cells after exposure to TGF-β1 with a linear gradient of 0.1–0.7 M NaCl in 8 M urea buffer. Dense cultures of bovine aortic endothelial cells were incubated at 37°C for 48 h with TGF-β1, with heparitinase generated only one prominent band of HSPG core protein, with a molecular mass of approximately 400 kDa, from the conditioned medium of the control and TGF-β1-exposed cells, respectively. The open and closed inverted triangles indicate the peak position of the low Mₗ subclass of CS/DSPGs isolated from the conditioned medium of the control and TGF-β₁-exposed cells, respectively.

DEAE-Sephadex peak II consists of HSPGs, and peak III is composed of CS/DSPGs. In the same experiments, the sizes of the GAG chains isolated from the subclasses of interest were estimated by Sepharose CL-6B chromatography (41) to determine if TGF-β₁ treatment altered the GAG chain length of CS/DSPGs and HSPGs. As shown in Table II, the low Mᵢ subclass of CS/DSPGs of dense endothelial cells bore longer chondroitin/dermatan sulfate chains after exposure to TGF-β₁. The size of the GAG chains synthesized by cells exposed to TGF-β₁ increased from approximately Mᵢ 39,000 to Mᵢ 60,000. However, the effect of TGF-β₁ on this class of PG is dependent on cell density, because the length of GAG chains borne by the low Mᵢ subclass of CS/DSPGs from the conditioned medium of the sparse cells was unchanged. In addition, the effect of TGF-β₁ on GAG chain length is specific to CS/DSPGs, since the size of heparan sulfate chains bound to either the high or the low Mᵢ subclass of HSPGs accumulated in the cell layer of dense cells was not significantly different in control and TGF-β₁-treated cultures (Table II). These results also suggest that the larger hydrodynamic size of the small CS/DSPGs in TGF-β₁-treated dense cultures is due to the presence of longer chondroitin/dermatan sulfate chains attached to the core proteins.

To examine whether the synthesis of specific PG core proteins is induced by TGF-β₁, proteins were metabolically labeled with [35S]-labeled amino acids and analyzed by SDS-polyacrylamide gel electrophoresis either without or with prior digestion with heparitinase or chondroitin ABC lyase (see Table II). HMW, the high Mᵢ subclass; LMW, the low Mᵢ subclass. The open and closed circles indicate the control and TGF-β₁ treatment, respectively. The open and closed inverted triangles indicate the peak position of the low molecular weight subclass of CS/DSPGs isolated from the conditioned medium of the control and TGF-β₁-exposed cells, respectively.
A protein band had more incorporated radioactivity in extracts from TGF-β1-treated cells when compared with control cell extracts. Digestion with chondroitin ABC lyase generated CS/DSPG core protein bands with molecular masses of approximately 450, 105, 80, 70, 50, and 22 kDa from the conditioned medium. However, only the radioactivity of the 50-kDa CS/DSPG core protein was increased by TGF-β1. Similar results were obtained by the chondroitin ABC lyase digestion of extracts of the conditioned medium from sparse cell cultures (Fig. 9). The 400-kDa HSPG core protein from dense culture cell layer and the 50-kDa CS/DSPG core protein from the conditioned medium of dense and sparse cultures were identified by Western blotting of heparitinase- or chondroitin ABC lyase-digested samples, respectively (Figs. 8 and 9). The core protein of the large HSPG reacted with an anti-perlecan antibody, while the small CS/DSPG was identified as biglycan. Although Western blot analysis of the decorin core was performed, the protein was not detected in either dense or sparse endothelial cells (not shown). Western blotting agreed with the 35S-labeled amino acid incorporation data in that TGF-β1 markedly increased the immunostaining of perlecan core protein in the cell layer and biglycan core protein in the conditioned medium in dense endothelial cells. Similarly, TGF-β1 also increased the amount of biglycan core protein in the conditioned medium of the sparse cells (Fig. 9). These results clearly indicate that the synthesis and secretion of biglycan core protein by vascular endothelial cells are promoted by TGF-β1 in a cell density-independent manner; however, that of perlecan core protein is increased only when the cell density is high.

### TABLE II

| Proteoglycan source | $K_v$ value before digestion | $K_v$ value after digestion | Approximate chain size of GAGs | Sensitive to $M_r$ |
|---------------------|-----------------------------|-----------------------------|-----------------------------|--------------------|
| Dense cells         |                             |                             |                             |                    |
| High $M_r$ subclass of peak II from the cell layer |                             |                             |                             |                    |
| Control             | $V_0$                      | 0.250                       | 74,000                      | Heparitinase       |
| TGF-β1              | $V_0$                      | 0.250                       | 74,000                      | Heparitinase       |
| Low $M_r$ subclass of peak II from the cell layer |                             |                             |                             |                    |
| Control             | 0.330                      | 0.368                       | 39,000                      | Heparitinase       |
| TGF-β1              | 0.320                      | 0.357                       | 41,000                      | Heparitinase       |
| Low $M_r$ subclass of peak III from the conditioned medium |                             |                             |                             |                    |
| Control             | $V_0$                      | 0.368                       | 39,000                      | Chondroitin ABC lyase |
| TGF-β1              | $V_0$                      | 0.289                       | 60,000                      | Chondroitin ABC lyase |
| Sparse cells        |                             |                             |                             |                    |
| Low $M_r$ subclass of peak III from the conditioned medium |                             |                             |                             |                    |
| Control             | $V_0$                      | 0.368                       | 39,000                      | Chondroitin ABC lyase |
| TGF-β1              | $V_0$                      | 0.368                       | 39,000                      | Chondroitin ABC lyase |

FIG. 8. Accumulation of perlecan and biglycan core proteins labeled with 35S-labeled amino acids in the cell layer and the conditioned medium of dense vascular endothelial cells after exposure to TGF-β1. Dense culture of bovine aortic endothelial cells were incubated at 37 °C for 48 h with TGF-β1 (10 ng/ml) in the presence of 35S-labeled amino acids. Samples were run on a 4–12% gradient slab gel before and after digestion with heparitinase and chondroitin ABC lyase. Separately, heparitinase- and chondroitin ABC lyase-generated core proteins were probed with an antibody specific for perlecan and biglycan, respectively.

FIG. 9. Accumulation of biglycan core protein labeled with 35S-labeled amino acids in the conditioned medium of sparse vascular endothelial cells after exposure to TGF-β1. Sparse culture of bovine aortic endothelial cells were incubated at 37 °C for 48 h with TGF-β1 (10 ng/ml) in the presence of 35S-labeled amino acids. Samples were run on a 4–12% gradient slab gel before and after digestion with chondroitin ABC lyase. Separately, chondroitin ABC lyase-generated core proteins were probed with an antibody specific for biglycan.
DISCUSSION

The results of this study indicated that the regulation of the synthesis of specific PGs by cultured vascular endothelial cells after exposure to TGF-β1 is dependent upon cell density. Specifically, while TGF-β1 treatment increases the accumulation of biglycan in the conditioned medium of both sparse and dense cultures, perlecan deposition was selectively increased only in dense cultures. These changes were reflected both in the accumulation of heparan sulfate GAGs in the cell layer of dense cultures and chondroitin/dermatan sulfate chains in the conditioned medium of cultures of both cell densities. Similarly, TGF-β1 increases the accumulation of perlecan core protein only in the cell layer of the dense cells and of biglycan core protein in the medium of both the dense and sparse cells, as assessed both by metabolic labeling and Western blotting. In addition to a differential effect on PG protein expression, TGF-β1 promotes the elongation of chondroitin/dermatan sulfate chains bound to biglycan cores only in cultures of dense cells, while chondroitin/dermatan sulfate chains of biglycan in sparse cell cultures are not affected. As a result, the hydrodynamic size of biglycan is larger in TGF-β1-treated cultures of dense cells when compared with the size of biglycan in control cultures or TGF-β1-treated sparse cultures. The effect of TGF-β1 on GAG chain elongation appears to be specific to chondroitin/dermatan sulfate chains, because the length of the heparan sulfate chains of perlecan were unaffected by TGF-β1 treatment in either dense or sparse cultures. Taken together, these results indicate that exposure of dense endothelial cell cultures to TGF-β1 increases the synthesis and deposition of perlecan without a change in length of their heparan sulfate chains and increases the secretion of biglycan with longer chondroitin/dermatan sulfate chains in the conditioned medium of cultures of both cell densities. Thus, the regulation by TGF-β1 of the synthesis of perlecan and biglycan and the elongation of chondroitin/dermatan sulfate chains of biglycan is dependent on the cell density of vascular endothelial cell culture.

It has been reported that TGF-β1 regulates the synthesis and structure of PGs, especially CS/DSPGs, in various cell types, including mesenchymal and epithelial cell lines (23). In human fibroblasts, TGF-β1 was shown to stimulate the synthesis of versican and biglycan but not decorin (24, 25). However, in rat myocardial fibroblasts, the cytokine causes an increase in the synthesis of both biglycan and decorin with an elongation of the GAG side chains (26). In vascular smooth muscle cells, TGF-β1 promotes both the synthesis of core protein and the elongation of chondroitin/dermatan sulfate chains of versican (44) and biglycan (45) but does not affect the expression of the core protein of decorin, although the length of decorin GAG chains is increased (45). These results indicate that the regulation of synthesis and structure of individual PGs by TGF-β1 differs among different cell types. The present study agrees with previous work in that TGF-β1 promotes the synthesis of biglycan core protein and the elongation of chondroitin/dermatan sulfate chains by smooth muscle cells (45). Increase in perlecan expression by TGF-β1 is also observed in colon carcinoma cells (46). The novel finding that the cell density-dependent regulation of perlecan core protein synthesis and chondroitin/dermatan sulfate chain length, but not heparan sulfate chain elongation, by the cytokine may, however, be unique to the endothelial cell.

Although the mechanism for the cell density dependence of stimulation of perlecan and biglycan synthesis and structure by TGF-β1 is unclear, it has been shown that the effect of TGF-β1 on the growth of vascular smooth muscle cells is dependent on the cell density (36). The cytokine inhibits cell growth when the cell density is low but promotes proliferation when the cell density is high. Although cell growth may not be coupled directly to PG metabolism, synthesis of some PGs, such as the large chondroitin sulfate PG versican, as well as biglycan, are regulated in concert with growth stimulation (44, 45). Moreover, the deposition of some PGs may modify the subsequent response of cells to growth factor stimulation. For example, developmental induction of perlecan expression is negatively correlated with the acquisition of PDGF responsiveness (47) during vascular development, and substrate-bound perlecan inhibits the response of vascular smooth muscle cells to that growth factor in vitro (48). The heparan sulfate chains of perlecan, moreover, can bind heparin-binding growth factors, such as FGF-2, and regulate the activity of these growth factors (49). When complexed with heparan sulfate chains, FGF-2 is protected from proteolytic degradation (50, 51) and maintains a capacity for long term stimulation of endothelial cell functions (52) such as proliferation, migration (32), and plasminogen activator activity (32, 52). In addition to a capacity for long term storage of heparin-binding growth factors, the heparan sulfate chains on perlecan also exhibit relatively strong anti-thrombin III binding (7). TGF-β1 is released from aggregated platelets after vascular endothelial injury (17) and appears to regulate extracellular matrix formation by endothelial cells near the damaged sites. Our results suggest that proliferating cells in regenerating endothelium may not synthesize additional perlecan in response to stimulation by this cytokine. This modification of PG synthesis during the response to injury could slow the regeneration of the antithrombotic nature of the undamaged endothelial monolayer and promote the continued release of FGF-2, rather than its sequestration within the subendothelial matrix. In contrast, after regrowth of the endothelial cell monolayer, continued TGF-β1 activation may induce increased perlecan synthesis and deposition to enhance repair of the damaged site and a return to homeostasis. Therefore, cell density-dependent regulation of perlecan synthesis by TGF-β1 may aid in the sequential events required during the repair of the injured endothelium.

In the present study, TGF-β1 was found to promote the synthesis of biglycan by both dense and sparse endothelial cells and increase the size of chondroitin/dermatan sulfate chains of biglycan synthesized by dense cells. Monolayers of vascular endothelial cells express biglycan but not decorin (10), although decorin is expressed during in vitro angiogenesis (12). Biglycan and decorin are closely related small leucine-rich CS/DSPGs (53, 54) and have been found in the arterial wall (11, 19, 55–60). The core proteins of both biglycan and decorin bind TGF-β and sequester the cytokine from the cell surface receptor (33–35). It has been proposed previously that the up-regulation of small leucine-rich PG synthesis by TGF-β1 may serve as a negative feedback loop to limit TGF-β1 activity (45), and our results are also consistent with this suggestion. An additional consequence of TGF-β1-driven biglycan synthesis may be inferred from the ability of CS/DSPGs to bind lipoproteins. In atherosclerotic plaques, low density lipoprotein is bound to chondroitin/dermatan sulfate chains (61), and the formation of GAG-low density lipoprotein complexes enhances the accumulation of low density lipoprotein by macrophages (62). Also, biglycan has been co-localized with apolipoprotein E in human atherosclerotic lesion (63). The present study suggests that biglycan may accumulate in the subendothelial extracellular matrix in response to TGF-β. Although resident smooth muscle cells probably synthesize most of the biglycan within the vessel wall, enhanced biglycan expression by endothelial cells at sites of vascular injury may influence the binding of lipoproteins within the intima and modify the progression of
atherosclerosis.

This work demonstrates that the regulation of perlecan and biglycan synthesis by TGF-β1 in vascular endothelial cells is dependent on cell density. The cell density-dependent regulation by TGF-β1 of synthesis and structure of endothelial cell PGs may be involved in the regulation of endothelial cell functions by growth factors and cytokines and in the increased accumulation of extracellular matrix during repair of vascular injury.

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