Original Article

Transforming Growth Factor-β2 and Connective Tissue Growth Factor in Proliferative Vitreoretinal Diseases

Possible Involvement of Hyalocytes and Therapeutic Potential of Rho Kinase Inhibitor

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The critical association of connective tissue growth factor (CTGF), which is thought to be one of the downstream mediators of transforming growth factor-β (TGF-β), with vitreoretinal diseases remains to be clarified. In the current study, we first demonstrated the correlation between the concentrations of TGF-β2 as well as CTGF in the vitreous and CTGF gene regulation in cultured hyalocytes. Concentrations of TGF-β2 and CTGF in the vitreous from patients with proliferative vitreoretinal diseases were significantly higher than in those with nonproliferative diseases, and there was a positive correlation between their concentrations ($r = 0.320$, $P < 0.01$). Cultured hyalocytes expressed CTGF mRNA, which was enhanced in the presence of TGF-β2, associated with nuclear accumulation of Smad4. TGF-β2–dependent Smad4 translocation and CTGF gene expression were mediated through Rho kinase and at least partially via p38 mitogen-activated protein kinase. Finally, fasudil, a Rho kinase inhibitor already in clinical use, inhibited both Smad4 translocation and CTGF gene expression. In conclusion, combined effects of TGF-β2 and CTGF appear to be involved in the pathogenesis of proliferative vitreoretinal diseases. Hyalocytes may be a possible source of CTGF and thus might play a role in vitreoretinal interface diseases. Furthermore, Rho kinase inhibitors might have therapeutic potential to control fibrotic disorders in the eye. *Diabetes* 56:231–238, 2007

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CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; RRD, rhegmatogenous retinal detachment; TGF-β, transforming growth factor-β.

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Contraction and remodeling of matrix are important elements of the tissue repair process; however, excessive contraction induces pathological scarring in a variety of tissues, such as skin (1) and the eye (2,3). Despite the dramatic development of vitreoretinal surgery, proliferative vitreoretinal diseases such as proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR) still remain common causes of severe visual loss or blindness (4,5). Better understanding of the pathogenesis of proliferative vitreoretinal diseases is thus needed for improved management of the disease. One of the critical causes of blindness by PVR and PDR is tractional retinal detachment caused by the formation of contractile preretinal fibrous membranes. The membrane formation is characterized by proliferation and migration of the cells and excessive synthesis and deposition of extracellular matrix proteins (6).

The tissue repair process is regulated by a number of polypeptides, including cytokines and growth factors. Transforming growth factor-β (TGF-β) is a multifunctional cytokine regulating a variety of pivotal biological responses, including cell growth, differentiation, apoptosis, migration, immune cell function, production of extracellular matrix proteins, and collagen gel contraction (7,8). The multiple biological actions of TGF-β thus play a central role in many fibrotic diseases (9) and are also presumed to play an important role in the pathological mechanisms of proliferative vitreoretinal diseases. The activity of TGF-β is actually known to be elevated in eyes associated with PVR or PDR, and TGF-β correlates with intraocular fibrosis (10,11). However, little is known about the downstream mediators of this effect.

Connective tissue growth factor (CTGF), also known as CCN2, is a 38-kDa cysteine-rich polypeptide that was originally identified from conditioned medium of human umbilical vein endothelial cells (12). CTGF induces the production of extracellular matrix, such as collagen and fibronectin, and causes fibrosis (13). In fact, expression of CTGF is promoted in many fibrotic diseases, such as scleroderma (1) and cirrhosis of the liver (14). In some kinds of cells, such as fibroblasts, mesangial cells, and endothelial cells, because of findings showing that CTGF is strongly induced by TGF-β and that TGF-β and CTGF share many biological functions in fibrosis, CTGF is con-

sidered to be a downstream mediator of TGF-β (15,16). It has been recently suggested that CTGF is involved in the pathogenesis of PVR and PDR according to the reports that CTGF is overexpressed in the membranes of eyes with PVR (17) or PDR (18), but the origin of CTGF and its expressional regulation still remain to be clarified. Whereas retinal pigment epithelial cells are considered to be a key element in the development of proliferative vitreoretinal diseases, previous studies, including our own, revealed that other types of cells, such as hyalocytes, glial cells, vascular endothelial cells, and fibroblast-like cells, also appeared to play a crucial role (6,19,20).

Hyalocytes are the cells located in the cortical vitreous abutting the inner surface of the retina (21). Morphological studies have demonstrated that hyalocytes, which possess lysosome-like granules, mitochondria, and micropinocytotic vesicles in their cytoplasm, belong to the monocyte/macrophage lineage (22–24). They are considered to be originated from bone marrow and to regenerate in the vitreous cavity (25), as well as to be actively associated with the maintenance of vitreous transparency under physiological conditions (26,27). In contrast, it is also thought that the hyalocytes themselves are involved in vitreoretinal interface diseases, such as epiretinal membrane formation, macular hole, and diabetic macular edema (28,29). It was reported that human hyalocytes in diabetic eyes have a different shape compared with those in normal eyes, and their number seemed to be increased (30). We hypothesized that hyalocytes might be one of the sources of CTGF and may play a role in the formation of fibrotic membrane in proliferative vitreoretinal diseases such as PVR and PDR.

Several recent studies have shown that CTGF gene expression might partially interact with Rho signaling pathways and mitogen-activated protein kinase (MAPK) pathways (31,32). Rho proteins belong to a family of small GTPases (RhoA, RhoB, RhoC, Rac-1, and cdc42) that regulate a wide variety of cellular functions, including cell adhesion, formation of stress fibers, and cellular contractility through actin cytoskeletal reorganization (33). Modulation of these cellular functions by Rho proteins largely depends on the activation of their downstream effector Rho kinase. The P38 MAPK pathway is activated by stress stimuli, inducing apoptosis, but in some cellular systems, it has been implicated in proliferation and differentiation (34). The P44/p42 MAPK pathway is mostly involved in proliferation and differentiation and is generally considered antiapoptotic (35). To investigate whether these pathways participate in TGF-β2–dependent CTGF mRNA expression and its regulation by hyalocytes, we also examined the effects of potent and selective inhibitors of each pathway. Furthermore, we examined the effects of lysophosphatidic acid, an agonist of the Rho signaling pathway (33), on CTGF gene expression and its regulation. Finally, we investigated the therapeutic potential of fasudil, a Rho kinase inhibitor, which has already been in clinical use for the inhibition of cerebrovascular spasm.

RESEARCH DESIGN AND METHODS

Recombinant human TGF-β2 and CTGF were purchased from Sigma-Aldrich (Tokyo) and PeproTech (London), respectively. Y27632, SB203580, PD98059, and lysophosphatidic acid were purchased from Calbiochem. A mouse monoclonal antibody against Smad4 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho-p38 MAPK, p38 MAPK, phospho-p44/p42 MAPK, and p44/p42 MAPK were obtained from Cell Signaling. Horseradish peroxidase–conjugated secondary antibody was obtained from Bio-Rad (Hercules, CA). Fasudil, a potent and selective Rho-kinase inhibitor, was kindly provided by Asahi Kasei Pharma (Tokyo).

Enzyme-linked immunosorbent assay. This study was carried out with approval from an institutional review board and performed in accordance with the ethical standards of the 1989 Declaration of Helsinki. We obtained written informed consent from the patients. Vitreous samples were collected from patients who underwent pars plana vitrectomy because of nonproliferative vitreoretinal diseases (macular hole and rhegmatogenous retinal detachment [RRD]) and proliferative vitreoretinal diseases (PDR and PVR). Concentrations of TGF-β2 in the vitreous were measured by a human TGF-β2 immunoassay kit (R&D Systems) according to the manufacturer's protocols. To detect CTGF in the vitreous, we performed sandwich enzyme-linked immunosorbent assays with two different anti-human CTGF antibodies, as previously reported (36). Enzyme-linked immunosorbent assay plates (96 wells; Falcon) were coated with 100 μl of 1 μg/ml goat polyclonal anti-human CTGF antibody, which had an epitope within the internal region of CTGF (Santa-Cruz), in 0.1 mol/l carbonate (pH 9.5) overnight at 4°C. After washing, unbound sites were blocked by incubation with 200 μl of PBS containing 1% BSA for 2 h at 37°C. After washing with 200 μl of PBS three times, 100 μl of vitreous samples and recombinant human CTGF as standard were added to the well and incubated for 2 h at room temperature. After washing three times, we added 100 μl of 1 μg/ml monoclonal anti-human CTGF COOH-terminal peptide antibody (R&D systems) in PBS containing 0.01% BSA. The plates were incubated for 2 h at room temperature and subsequently washed three times, and 100 μl of 1:50 dilution EnVision+ (DakoCytomation, Carpinteria, CA), anti-mouse horseradish peroxidase–labeled polymer, with PBS containing 0.01% BSA was added. After a 1-h incubation, plates were washed three times and incubated with 100 μl of OPD (1,2-phenylenediamine, dihydrochloride) solution for 30 min in the dark, and the intensity of the wells was determined at 490 nm (Immuno Mini NJ-2300; Nalge Nunc International).

Cell culture. Bovine eyes were obtained from a local abattoir. The posterior part of the vitreous body was extracted and washed once in Dulbecco's modified Eagle's medium (DMEM; Sigma). The vitreous was chopped into several pieces and incubated in type I collagen–coated dishes in DMEM containing 10% fetal bovine serum (Invitrogen-Gibco, San Diego, CA) for 1 week. The cells that proliferated on the dishes were then subcultured in type I collagen–coated dishes in DMEM supplemented with 10% fetal bovine serum. Cultured hyalocytes obtained between passages 4 and 7 that had shown no obvious morphological changes were used in the following experiment. Isolated cells were immnocytochemically identified as hyalocytes expressing S-100 protein, but neither expressed glial fibrillary acidic protein nor cytokeratin, as previously described (37). Bovine retinal pigment epithelial cells and retinal capillary endothelial cells were also isolated from bovine eyes and cultured as previously reported (38,39). MIO-M1, a cell line of the human Muller cell, was a kind gift from Dr. G.A. Limb, Division of Cell Biology, Institute of Ophthalmology, London.

Detection of CTGF mRNA by Northern blot analysis. Northern blot analysis was performed as previously described (38). Subconfluent hyalocytes, bovine retinal pigment epithelial cells, retinal capillary endothelial cells, and MIO-M1 under normal growth medium conditions were collected, and total RNA was extracted by the AGPC (acid guanidine thiocyanate-phenol-chloroform) extraction method. Radioactive CTGF cDNA probe was generated using a labeling kit (Multiprime; Amersham) and [^{32}P]dCTP (NEN, Boston, MA). Quantitation of Northern blot analysis was performed using a computing phosphorescence imager (PhosphorImager with ImageQuant software analysis; Molecular Dynamics, Sunnyvale, CA). Lane loading differences were normalized by rehybridization with rediolabeled 36B4 cDNA probe as an internal control gene.

Detection of Smad4 protein in the nuclei by Western blot analysis. Nuclear extracts were isolated from hyalocytes with a CelLytic NuCLEAR extraction kit (Sigma) according to the manufacturer's protocols. Protein samples were separated by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes (New England Biolabs, Beverly, MA). The blots were blocked with skim milk and incubated overnight at 4°C with antibody against Smad4 (1:1,000). After being washed with t-TBS (20 mmol/l Tris, pH 7.5, 500 mmol/l NaCl, and 0.1% Tween-20) three times for 10 min each, the membranes were incubated with horseradish peroxidase–labeled rabbit anti-goat IgG (1:4,000; Bio-Rad, Richmond, CA) for 30 min at room temperature. Visualization was performed with an enhanced chemiluminesence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's protocols.

Rho activation assay. Rho activity in hyalocytes under the presence or absence of TGF-β2 was analyzed using a Rho activation assay kit (Upstate Biotechnology). Briefly, subconfluent hyalocytes were starved and unstimulated or stimulated with TGF-β2 (0.3 nmol/l) for indicated periods of time (5, 15, 30, and 60 min), and cell lysates were prepared according to the manufacturer's protocols. After centrifugation, supernatants were incubated

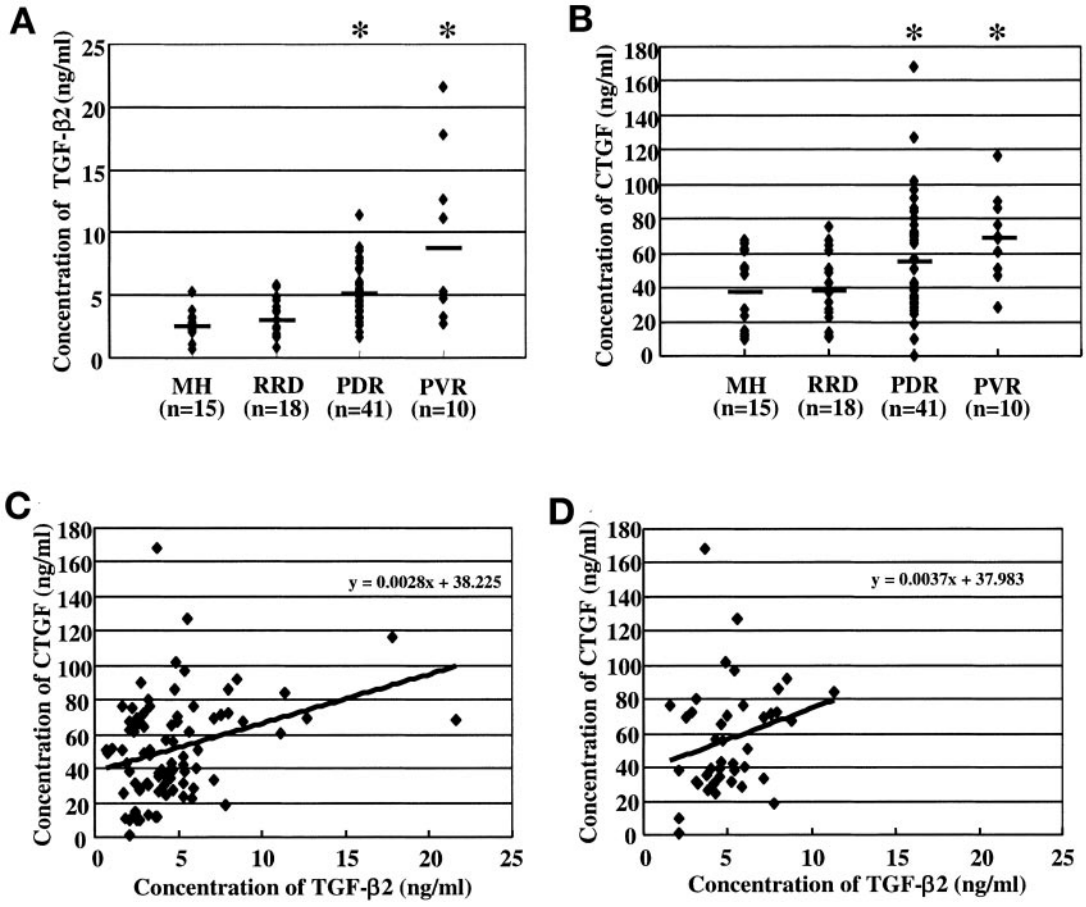

FIG. 1. Concentrations of TGF-β2 and CTGF in the vitreous. Vitreous samples were collected from patients with nonproliferative vitreoretinal diseases (macular hole and RRD) and proliferative vitreoretinal diseases (PDR and PVR). *A*: Concentrations of TGF-β2 in the vitreous were measured by enzyme-linked immunosorbent assay (macular hole: $n = 8$; RRD: $n = 7$; PDR: $n = 39$; and PVR: $n = 4$). *$P < 0.01$ compared with macular hole and RRD, respectively. *B*: Concentrations of CTGF in the vitreous were measured by sandwich enzyme-linked immunosorbent assay (same samples as in *A*). *$P < 0.01$ compared with macular hole and RRD, respectively. *C*: Statistical analysis for the correlation between them was performed ($r = 0.320$, $P = 0.0028$). *D*: PDR samples were extracted from all samples, and then statistical analysis for the correlation was performed again ($r = 0.238$, $P = 0.134$). MH, macular hole.

with rhotekin Rho-binding peptide immobilized on agarose, and activated GTP-Rho bound to rhotekin-agarose was detected by Western blotting with anti-Rho antibody.

Phosphorylation of p38 MAPK and p44/p42MAPK. Hyalocytes were starved and untreated or treated with TGF-β2 (0.3 nmol/l) for the indicated time (2, 5, 15, 30, and 60 min), and the cells were lysed with 1× Laemmli buffer (50 mmol/l Tris, pH 6.8, 2% SDS, and 10% glycerol) containing protease inhibitor and phosphatase inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l NaF, and 0.5 mmol/l Na_3VO_4). Western blot analysis was performed as described above with antibodies against phospho-p38 MAPK (1:1,000) and phospho-p44/p42 MAPK (1:1,000), respectively. Lane loading differences were normalized by reblotting the membranes with antibodies against p38 MAPK (1:1,000) and p44/p42 MAPK (1:1,000), respectively.

Statistical analysis. The experimental data are expressed as the means ± SD. $P < 0.05$ was considered statistically significant using Student's t test in normally distributed populations. The correlation between the differences in CTGF and the differences in TGF-β2 was statistically analyzed using StatView version 5.0 (SAS Institute, Cary, NC), and statistical significance was assumed at $P < 0.05$.

RESULTS

Concentrations of TGF-β2 and CTGF protein in the vitreous. Average concentrations of TGF-β2 protein in the vitreous were 2.63 ng/ml (macular hole, $n = 15$), 3.24 (RRD, $n = 18$), 5.09 (PDR, $n = 41$), and 8.54 (PVR, $n = 10$). Concentrations of TGF-β2 with PDR or PVR were significantly higher than those with macular hole and RRD, respectively ($P < 0.01$) (Fig. 1*A*). Average concentrations of CTGF protein in the vitreous were 38.90 ng/ml (macular hole, $n = 15$), 39.87 (RRD, $n = 18$), 56.63 (PDR, $n = 41$), and 69.47 (PVR, $n = 10$). Concentrations of CTGF in eyes with PDR or PVR were also significantly elevated compared with those with macular hole and RRD, respectively ($P < 0.01$) (Fig. 1*B*). In addition, there was a significant correlation between the concentrations of TGF-β2 and CTGF ($r = 0.320$, $P = 0.0028$, $n = 84$) (Fig. 1*C*), whereas there was no significant correlation between their concentrations in the vitreous with PDR ($r = 0.238$, $P = 0.134$, $n = 41$) (Fig. 1*D*).

Various ocular cell types express CTGF mRNA. As shown in Fig. 2, all of the bovine hyalocytes, bovine retinal pigment epithelial cells, bovine retinal capillary endothelial cells, and Muller cells (MIO-M1) expressed CTGF mRNA under normal culture conditions. These results indicated that various cell types could be the sources of CTGF in the eye.

Expression of CTGF mRNA and nuclear translocation of Smad4 protein. We investigated whether the expression of CTGF mRNA by hyalocytes is promoted in the presence of TGF-β. The cells were starved and left unstimulated or stimulated with TGF-β2 (0.3 nmol/l) for 1, 4, 10, and 24 h, and then total RNA was extracted for Northern blot analysis. As shown in Fig. 3*A*, CTGF mRNA

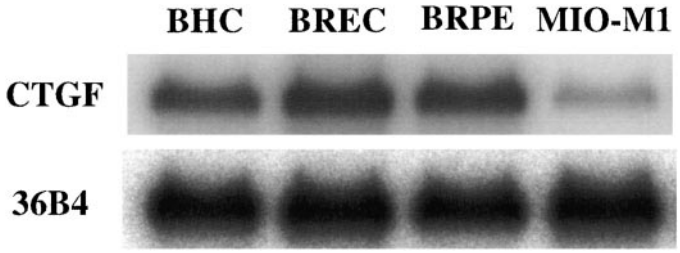

FIG. 2. CTGF gene expression by different types of cells in the posterior segment of the eye. CTGF gene expression by bovine hyalocytes (BHC), retinal pigment epithelial cells (BRPE), capillary endothelial cells (BREC), and Muller cells (MIO-M1) under normal culture conditions were examined by Northern blot analysis. Lane loading differences were normalized by rehybridization with rediolabeled 36B4 cDNA probe as an internal control gene.

expression was promoted by TGF-β2 and reached its maximum expression level at 10 h after stimulation (4.05-fold compared with control, $P < 0.01$) and was maintained at least up to 24 h.

We also examined the levels of Smad4 protein in the nuclei in the presence of TGF-β2 to investigate the intracellular signaling pathway of hyalocytes. Subconfluent hyalocytes were starved and unstimulated or stimulated with TGF-β2 (0.3 nmol/l) for 15, 30, 60, 120, and 180 min. Although the amount of Smad4 protein in the nuclei was low in the quiescent cells, its concentration was significantly elevated by TGF-β2 treatment within 15 min and was maintained at least 180 min ($P < 0.01$) (Fig. 3*B*).

TGF-β2 causes Rho activation and p38 MAPK phosphorylation. To examine whether TGF-β2 activates the Rho/Rho kinase, p38 MAPK, and p44/p42 MAPK pathways in hyalocytes, we investigated Rho activation, p38 MAPK phosphorylation, and p44/p42 MAPK phosphorylation in hyalocytes in the presence of TGF-β2. Levels of Rho-GTP were elevated by TGF-β2 initially after 15 min of stimulation and were sustained even after 60 min of stimulation (Fig. 4*A*). Phosphorylation of p38 MAPK in the presence of TGF-β2 was observed from 30 min of stimulation and maintained at least up to 60 min (Fig. 4*B*), whereas p44/p42 MAPK was not phosphorylated by TGF-β2 at any stimulation time from 0 to 60 min (Fig. 4*C*).

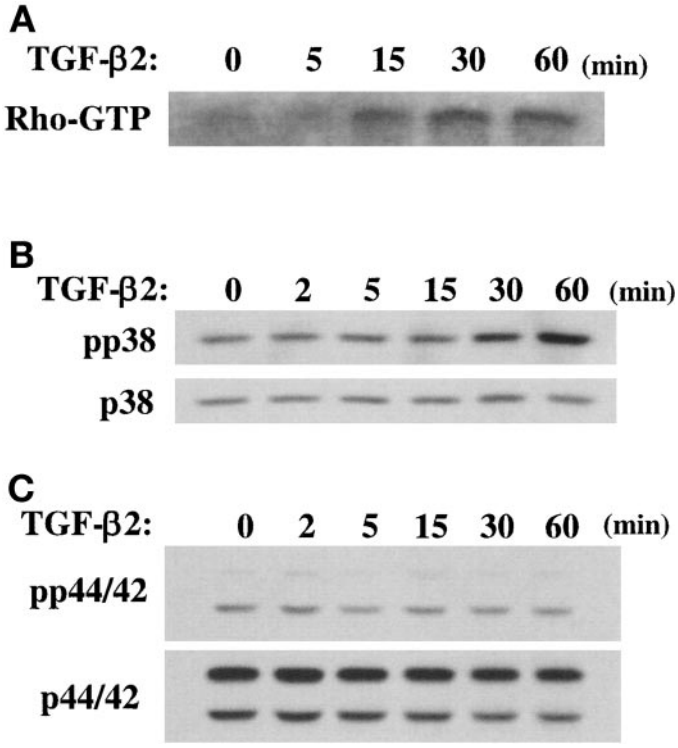

FIG. 4. TGF-β2 induced Rho activity and p38 MAPK phosphorylation but did not induce p44/p42 MAPK phosphorylation. Hyalocytes were starved and unstimulated or stimulated with TGF-β2 (0.3 nmol/l) for the indicated periods of time. *A*: The levels of Rho-GTP in the presence of TGF-β2 were detected by Rho pull-down assay. *B*: Total cell lysates were subjected to Western blot analysis with an antibody against phosphorylated p38 MAPK (pp38 MAPK). Lane loading differences were normalized by reblotting the membrane with an antibody against p38 MAPK. *C*: Phosphorylated p44/p42 MAPK (pp44/42 MAPK) and p44/p42 MAPK were also examined in the same manner as in *B*.

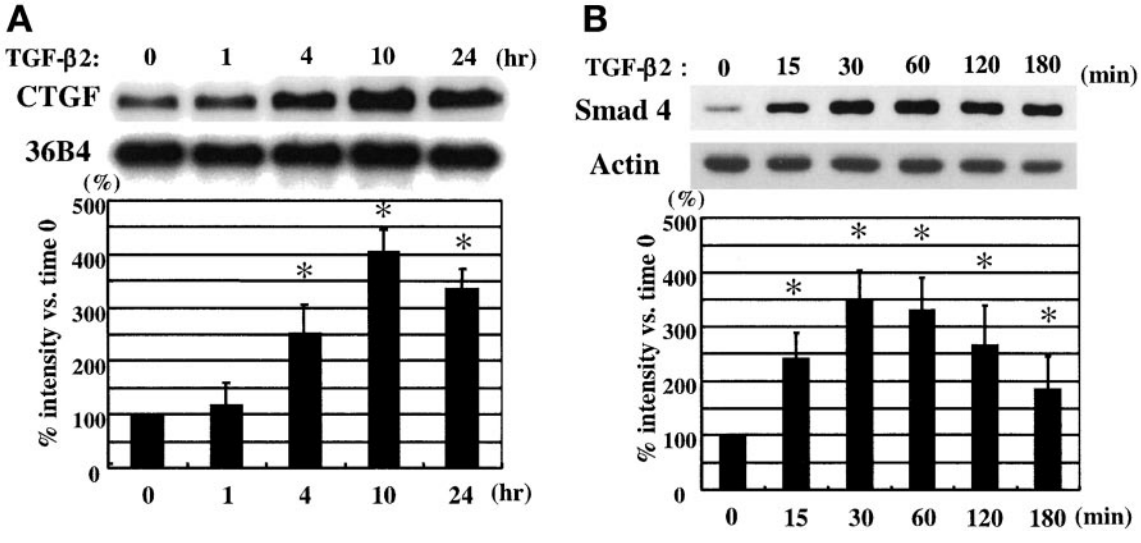

FIG. 3. CTGF mRNA expression and nuclear translocation of Smad4 by hyalocytes in the presence of TGF-β2. Hyalocytes were starved and then untreated or treated with TGF-β2 (0.3 nmol/l) for the indicated time. *A*: CTGF mRNA expression in the presence of TGF-β2 was analyzed by Northern blot analysis. Lane loading differences were normalized by rehybridization with rediolabeled 36B4 cDNA probe as an internal control gene. A representative blot from three independent experiments is demonstrated. Signal intensities were quantified and are expressed as a percentage by the ratio of CTGF to 36B4 at time 0. *B*: Nuclear extracts were isolated, and the levels of Smad4 protein were analyzed by Western blot analysis. Values were normalized to actin levels. Signal intensities were quantified and are expressed as a percentage by the ratio of Smad4 to actin at time 0. *$P < 0.01$ compared with time 0.

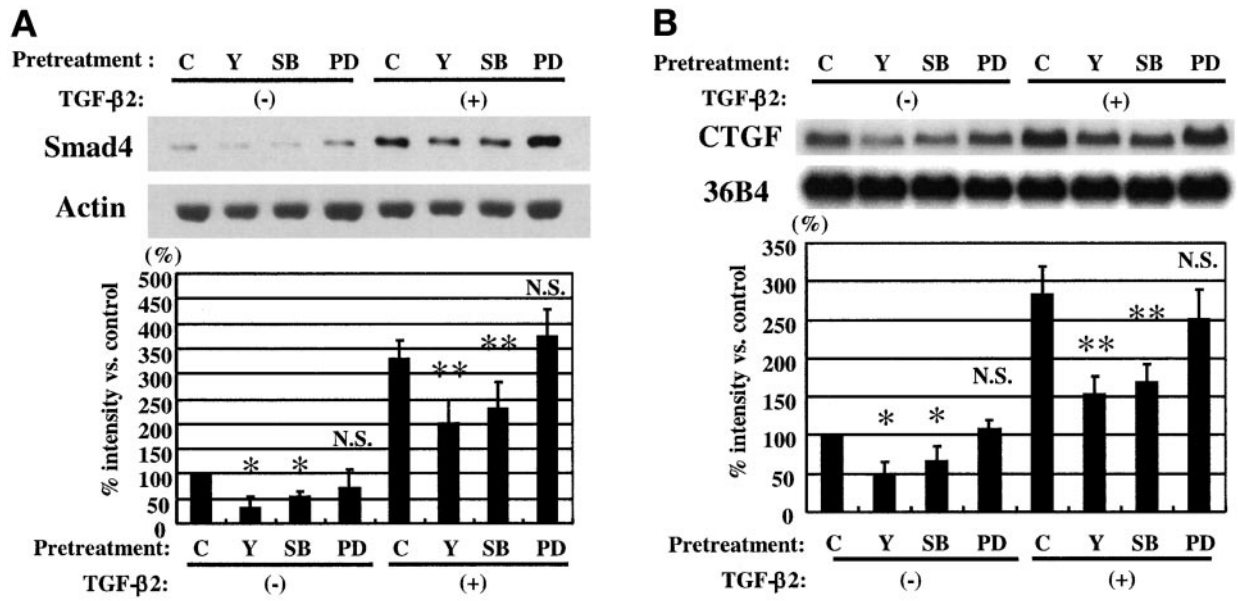

FIG. 5. Effects of Y27632, SB203580, and PD98059 on TGF-β2–dependent nuclear accumulation of Smad4 and CTGF gene expression. After starvation, hyalocytes were pretreated for 30 min with or without Y27632 (10 μmol/l), SB203580 (20 μmol/l), or PD98059 (20 μmol/l), potent and selective inhibitors of Rho kinase, p38 MAPK, and p44/p42 MAPK, respectively, and subsequently treated with TGF-β2 (0.3 nmol/l). *A*: After pretreatment, hyalocytes were stimulated with TGF-β2 for 45 min. Nuclear extracts were isolated, and the levels of Smad4 were analyzed by Western blot analysis. **P* < 0.05 compared with control/TGF-β2$^-$; ***P* < 0.05 compared with control/TGF-β2$^+$. *B*: After pretreatment, the cells were stimulated with TGF-β2 for 24 h. The total RNA was subjected to Northern blot analysis for CTGF mRNA. Representative blots from three independent experiments are demonstrated, respectively. Signal intensities were quantified and expressed as a percentage by the ratio of Smad4 to actin or CTGF/36B4 under the condition of absence of C/TGF-β2. **P* < 0.05 compared with control/TGF-β2$^-$; ***P* < 0.01 compared with control/TGF-β2$^+$. C, control buffer; N.S., nonsignificant; PD, PD98059; SB, SB203580; Y, Y27632.

Y27632 and SB203580 suppressed TGF-β2–dependent nuclear translocation of Smad4 protein and CTGF gene expression. We confirmed the regulation of TGF-β2–dependent CTGF gene expression by the Rho/Rho kinase, p38 MAPK, or p44/p42 MAPK pathways. We examined the effect of Y27632, SB203580, and PD98059, potent and selective inhibitors of Rho kinase, p38 MAPK, and p44/p42 MAPK, respectively. As shown in Fig. 5*A* and *B*, Y27632 and SB203580 significantly suppressed nuclear accumulation of Smad4 protein and CTGF gene expression in both the absence and presence of TGF-β2 ($P < 0.05$). On the other hand, PD98059 had no significant effect on either nuclear accumulation of Smad4 protein or CTGF gene expression. Our results suggest that the suppressive effect of Y27632 and SB203580 on the expression of CTGF mRNA is mediated via the inhibition of nuclear translocation of Smad4 protein.

Lysophosphatidic acid promoted nuclear accumulation of Smad4 and the expression of CTGF mRNA. To confirm the involvement of the Rho signaling pathway in CTGF gene expression, the effects of lysophosphatidic acid, an agonist of the Rho signaling pathway, on nuclear translocation of Smad4 and the expression of CTGF mRNA were examined. Levels of Smad4 in the nuclei and expression of CTGF mRNA were significantly promoted in the presence of lysophosphatidic acid (1.71- and 1.79-fold compared with respective controls) (Fig. 6*A* and *B*), suggesting that the Rho kinase pathway lies, at least in part, upstream of Smad pathway.

Fasudil prohibited TGF-β2–dependent nuclear translocation of Smad4 and CTGF gene expression. We further examined the effect of fasudil, a Rho kinase inhibitor that has already been in clinical use for the inhibition of cerebrovascular spasm, on Smad pathway and CTGF mRNA expression. The concentration of Smad4 in the nuclei rose in the presence of TGF-β2, whereas the effect was significantly suppressed by fasudil in a dose-dependent manner (Fig. 7*A*). Similarly, CTGF mRNA

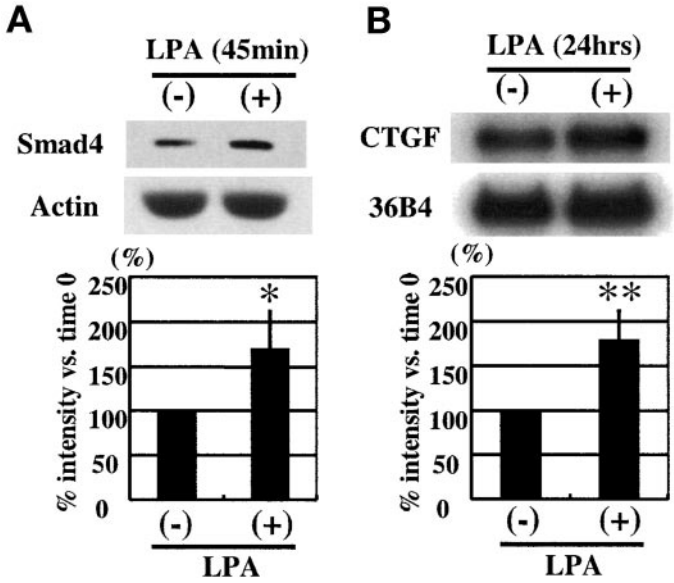

FIG. 6. Lysophosphatidic acid (LPA) promoted nuclear translocation of Smad4 and the expression of CTGF mRNA. *A*: Starved hyalocytes were untreated or treated with 10 μmol/l of lysophosphatidic acid for 45 min, and then nuclear extracts were isolated, and the levels of Smad4 were examined by Western blot analysis. *B*: The cells were also unstimulated or stimulated with lysophosphatidic acid (10 μmol/l) for 24 h. Total RNA was extracted, and CTGF mRNA expression was analyzed by Northern blotting. Representative blots from four independent experiments are demonstrated, respectively. Signal intensities were quantified and are expressed as a percentage by the ratio of Smad4 to actin or CTGF to 36B4 at time 0. **P* < 0.05 and ***P* < 0.01 compared with absence of lysophosphatidic acid.

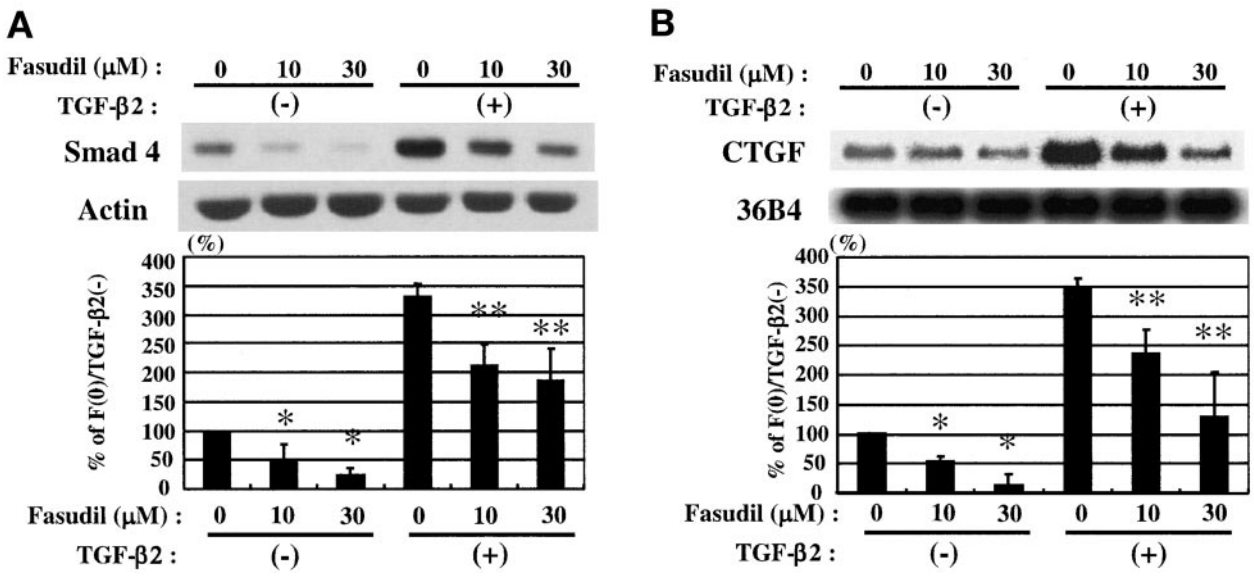

FIG. 7. Suppressive effect of fasudil on TGF-β2–dependent nuclear translocation of Smad4 and CTGF mRNA expression. Starved hyalocytes were untreated or pretreated with 10 or 30 μmol/l fasudil for 30 min. *A*: Pretreated hyalocytes were stimulated with TGF-β2 (0.3 nmol/l) for 45 min. Nuclear extracts were isolated and subjected to Western blot analysis for the detection of Smad4. *B*: The cells were stimulated with 0.3 nmol/l TGF-β2 for 24 h. CTGF gene expression was examined by Northern blot analysis. The membranes were rehybridized with 36B4 for normalization. Signal intensities were quantified and are expressed as a percentage by the ratio of Smad4 to actin or CTGF to 36B4 under the condition of fasudil(0)/TGF-β2(-). *$P < 0.01$ compared with fasudil(0)/TGF-β2(-); **$P < 0.01$ compared with fasudil(0)/TGF-β2(+). F, fasudil.

expression was significantly prohibited by fasudil in a dose-dependent manner in both the absence and presence of TGF-β2 (Fig. 7*B*).

DISCUSSION

In the current study, we demonstrated that concentrations of TGF-β2 and CTGF are elevated in the vitreous of eyes with proliferative vitreoretinal diseases (PDR and PVR), compared with those with nonproliferative diseases (macular hole and RRD), and that their concentrations are positively correlated. We further demonstrated that hyalocytes are one of the possible sources of CTGF, with expression upregulated in the presence of TGF-β2 mediated through Rho kinase and at least partially by p38 MAPK. In addition, our results indicated that fasudil might have therapeutic potential for the treatment of vitreoretinal interface diseases via inhibition of CTGF synthesis, at least in part.

Our findings of elevated concentrations of TGF-β2 and CTGF in the vitreous of eyes with proliferative vitreoretinal diseases and their positive correlation suggest their combinational role, at least in part, in the pathogenesis of proliferative vitreoretinal diseases. Although it is known that CTGF is a downstream mediator of TGF-β in vitro, this is the first demonstration of their correlation in vivo. Even though the mean concentrations of CTGF in PDR and PVR were higher than those of macular hole and RRD, respectively, the data showed some spread. It is possible that the concentrations depend on the activity of the diseases (e.g., active or quiescent PDR). It is also possible that the concentration of CTGF in the vitreous may be elevated before or during the formation of proliferative membranes and that afterwards, at a stage of contraction of the membrane (which is often the time of removal by vitreoretinal surgery), it may be lower. CTGF is known to promote extracellular matrix production and cause fibrosis, but it is difficult to ascertain with precision the onset time of fibrosis. In addition, even in simple RRD without fibrosis, which in the current study we classified in the control group, a recent study has revealed that the concentration of CTGF in subretinal fluid is increased with time between the diagnosis of retinal detachment and surgery (40). This suggests that long-standing RRD might predispose to PVR through CTGF and that the level of CTGF can be elevated even without morphologic fibrous change yet evident. Indeed, in this study, some RRD samples also had higher concentrations of CTGF compared with lower values in PDR or PVR. Further study is necessary to fully characterize the dynamics of CTGF expression in PDR or PVR. Moreover, the correlation between the concentrations of TGF-β2 and CTGF was significant but not so strong. This might have occurred because of the three TGF-β isoforms (TGF-β1, -β2, and -β3), we investigated only TGF-β2, even though it is the predominant isoform in the posterior segment of the eye (41). In addition, when only PDR samples were analyzed, there was no significant correlation between the concentrations of TGF-β2 and CTGF, and there was a better correlation between them in the other vitreous samples with macular hole, RRD, and PVR ($r = 0.400$, $P = 0.0074$, $n = 43$) (data not shown), and, despite low TGF-β2 concentrations, concentrations of CTGF in several vitreous samples with PDR were high. According to some recent studies, the expression of CTGF can be induced not only by TGF-β, but also by vascular endothelial growth factor (42), hypoxia (43), or high glucose (44). So it is possible that CTGF induced by vascular endothelial growth factor, for example, released from retinal endothelial cells (42) might diffuse into the vitreous independent of TGF-β in PDR eyes.

Although it was reported that CTGF has been overexpressed in preretinal membranes of eyes with PVR or PDR, as expected (17,18), the origin of CTGF and its regulation still remain to be clarified. We demonstrated in this study that hyalocytes, bovine retinal pigment epithelial cells, retinal capillary endothelial cells, and Muller cells can be

sources of CTGF. Hinton et al. (17) indicated that most CTGF-immunoreactive cells observed in PVR membrane were cytokeratin-positive cells associated with retinal pigment epithelial cells and also that most CTGF-immunoreactive cells observed in PDR membrane were smooth muscle actin–positive myofibroblast.

Recent studies have revealed that hyalocytes might be involved in the pathogenesis of vitreoretinal diseases (28,29). Our previous study revealed that hyalocytes could be transdifferentiated into myofibroblastic cells and come to express α-smooth muscle actin in the presence of TGF-β2 (9). We thus hypothesized that hyalocytes might be involved in the formation of fibrotic membrane via the expression of CTGF in proliferative vitreoretinal diseases. In the current study, we demonstrated that hyalocytes expressed CTGF and that its expression was significantly enhanced in the presence of TGF-β2, suggesting that the hyalocytes are one of the possible sources of CTGF synthesis in the vitreous cavity. Additionally, CTGF could stimulate the proliferation of cultured hyalocytes, suggesting that the hyalocytes themselves might be one of the elements of fibrous membrane (data not shown), consistent with our previous report demonstrating that transdifferentiated hyalocytes cause collagen gel contraction in the presence of TGF-β2 (9). The hyalocytes thus might participate in the initiation and progression of proliferative vitreoretinal diseases through the formation of proliferative membrane and its cicatricial contraction.

TGF-β signals through transmembrane receptor serine/threonine kinases to activate signaling intermediates called Smad proteins, which modulate the transcription of target genes (45). Eight kinds of Smad proteins were identified (Smad1–8) (46). TGF-β binding to its receptor causes Smad2/3 phosphorylation. Phosphorylated Smad2/3 then forms a complex with Smad4, and this complex translocates into the nuclei, where it regulates the transcription of target genes, such as CTGF, as a transcription factor. In the current study, we also demonstrated that TGF-β2 caused nuclear translocation of Smad4 in hyalocytes and that Smad2/3 was translocated into the nuclei after stimulation with TGF-β2 in the same manner as Smad4 (data not shown). These data suggest that enhanced expression of CTGF in the presence of TGF-β2 by hyalocytes might be caused by activation of the Smad pathway, similar to that observed in other types of cells such as fibroblasts (47) and mesangial cells (48).

Some recent studies revealed cross talk between Rho and MAPK pathways and Smad signaling downstream of TGF-β. As reviewed by Javelaud and Mauviel (49), TGF-β has been shown in numerous cell types to activate all Rho and p38 and p44/p42 MAPKs, but the activation by TGF-β is cell type–dependent, and, furthermore, the activated Rho and MAPKs might be involved in the phosphorylation of Smad proteins and regulate either Smad transcriptional activity or capacity to translocate into the cell nucleus. In this study, we demonstrated that TGF-β2 induced Rho activity and phosphorylation of p38 MAPK, but it did not phosphorylate p44/p42 MAPK in hyalocytes. Y27632 and SB203580 could partially but significantly prohibit TGF-β2–dependent nuclear accumulation of Smad4 and CTGF mRNA expression. However, PD98059 did not suppress either of them, different from many other types of cells reported, such as fibroblasts and smooth muscle cells (49). Furthermore, Nishida et al. (50) reported that Rho/Rho kinase participates in p38 MAPK activation. We also confirmed Y27632 inhibits TGF-β2–dependent phosphorylation of p38 MAPK by hyalocytes (data not shown), suggesting that the Rho/Rho kinase pathway might lie upstream of the p38 MAPK pathway in TGF-β2–dependent CTGF gene regulation.

With the prospect of a clinical application, we further demonstrated the inhibitory effect of fasudil, a Rho kinase inhibitor that has already been in clinical use for the inhibition of cerebrovascular spasm, on Smad pathway and CTGF mRNA expression. The results suggested that fasudil might suppress aberrant fibrosis of proliferative tissue through the inhibition of CTGF gene expression. In addition, our previous study revealed that fasudil inhibits collagen gel contraction by hyalocytes and thus might have an inhibitory effect on the contraction of the fibrotic membrane (9). Taken together, fasudil might have therapeutic potential for proliferative vitreoretinal diseases. However, further studies, not only in vitro but also in vivo, are necessary to more fully evaluate the therapeutic potential of fasudil for clinical use in the management of proliferative vitreoretinal diseases.

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