Expression of Hyaluronan Synthase in Intraocular Proliferative Diseases: Regulation of Expression in Human Vascular Endothelial Cells by Transforming Growth Factor-β

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Purpose: To investigate the role of hyaluronan (HA) and elucidate the mechanisms that regulate the expression of hyaluronan-synthesizing enzymes in vascular endothelial cells (VECs) in intraocular proliferative diseases.

Methods: Cultured VECs were used. Hyaluronan synthase (HAS) expression was determined on the mRNA products obtained by reverse transcription polymerase chain reaction (RT-PCR). The effect of transforming growth factor-β1 (TGF-β1) and/or platelet-derived growth factor-BB (PDGF-BB) on HAS expression was examined by quantitative RT-PCR and Western blot analysis. HAS expression in intraocular proliferative membranes was observed by immunohistochemistry.

Results: Cultured VECs expressed the three HAS isoforms. Stimulation of VECs with TGF-β1 induced a marked increase in the expression level of HAS2 mRNA and protein. The stimulatory effect of PDGF-BB was less potent. A synergistic or additive effect between TGF-β1 and PDGF-BB-induced HA synthesis was not observed. Furthermore, HAS1 and HAS2 exhibited differential expression in VECs and non-VECs populating intraocular proliferative membranes.

Conclusions: The expression of each HAS isoform is regulated differently by growth factors and cytokines in VECs. Importantly, HA-synthesizing enzymes were expressed in cells populating proliferative membranes obtained from eyes of patients with proliferative vitreoretinal diseases, and thus may be key molecules in the events that control progression of the proliferative diseases.

Key Words: Hyaluronan synthase, normal human microvascular endothelial cells, proliferative diabetic retinopathy, proliferative vitreoretinopathy, transforming growth factor-β.

Introduction

Angiogenesis1–4 plays a critical role in a variety of pathological conditions, such as diabetic retinopathy and age-related macular degeneration. The process of angiogenesis can be divided into several steps: (1) activation of vascular endothelial cells by external stimuli, (2) digestion and migration of the basal lamina and interstitium by activated endothelial cells, (3) cellular differentiation and proliferation, (4) formation of new vascular channels, and (5) the regulation of the basal lamina. These steps are regulated by various factors, including growth factors, cytokines, and extracellular matrix (ECM) components.1–6

To determine the molecular mechanisms of new vessel formation in proliferative membranes, the interactions between the ECM components and growth factors should be investigated. Hyaluronan (HA), an ECM component, is a high molecular weight linear glycosaminoglycan
composed of repeating disaccharide units with the following structure: D-glucuronic acid β(1→3) N-acetylglucosamine β(1→4). The molecule is widely distributed in ocular tissues, skin, synovial fluid, cartilage, and other tissues. HA plays an important role in regulating the functional activities of cells, eg, vascular endothelial cells, and is involved in cell migration, cell proliferation, and cell differentiation.

HAS synthesis is catalyzed by membrane-bound enzymes, termed hyaluronan synthases (HAS). In all vertebrates so far investigated, only three HAS isoforms (HAS1, HAS2, and HAS3) have been identified. Although the three HAS isoforms show a 55–71% sequence similarity, they differ in their chromosomal locations and functions. Several studies have shown that the expression of HAS is regulated differently by growth factors and cytokines, suggesting that the regulation of HAS expression affects both the composition and the function of the ECM. Thus, examination of HAS synthesis may be a useful way to investigate the interaction between the ECM and growth factors in the pathogenesis of new blood vessel formation in proliferative membranes.

In the present study, we have investigated the expression and regulation of HAS in cultured endothelial cells. Furthermore, proliferative membranes obtained from patients’ eyes during vitrectomy were tested for the expression levels of HAS. We have also investigated the expression and regulation of HAS in cultured vascular endothelial cells in this study. In addition, proliferative vitreoretinal membranes were revealed to express HAS immunohistochemically. These results suggest that HAS is expressed in ocular tissue and may be involved in the pathogenesis of proliferative membrane formation, including new vessel formation.

**Materials and Methods**

**Cell Cultures of Normal Human Microvascular Endothelial Cells**

Normal human microvascular endothelial cells (HMVECs; Kurabo Industries, Osaka, in joint development with Cascade Biologies, Portland, OR, USA) were prepared and used according to the recommendations of the manufacturer. Cells were cultured in 60-mm culture dishes (Falcon, Lincoln Park, NJ, USA), which were coated with a cell-adhesive factor (Kurabo). Fetal bovine serum (5% vol/vol), 10 ng/mL human epidermal growth factor (hEGF), 5 ng/mL human fibroblast growth factor (hFGF-b), hydrocortisone, heparin, dibutyryl cyclic adenosine monophosphate (dbcAMP), gentamicin, and amphoteracin-B were added to HuMediaMvG (Kurabo) before beginning the cell culture. When cells reached about 80% confluency, they were subcultured with 0.025% trypsin/ethylene diaminetetraacetic acid (Kurabo). HMVECs between the 5th and 12th passages were used in all experiments.

**RT-PCR of HAS1, HAS2, HAS3 mRNA in HMVECs**

The cDNAs for human HAS1, HAS2 and HAS3 were generated by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the cultured HMVECs, and cDNA was produced using RT (Super Script II; Gibco, Grand Island, NY, USA). The degenerate oligonucleotide primers for RT-PCR were designed based on well-conserved amino acid sequences of human and mouse HAS isoforms, which included the catalytic region of each human HAS isoform. Sequences of the primers were as follows: 5′-CTCTGGGTTGGCCTTCAATGTTGA-3′ (sense), 5′-TA CTTGGTGAGCATGAGCCCAT-3′ (antisense), for HAS1; 5′-GAAAGGGTCTG(C/T)CAGTCTTATTGTTT-3′ (sense), 5′-TT(C/T)CTCTCC(AGT)GTGGTCCCCCAA-3′ (antisense), for HAS2; 5′-ATGTACCAGCAACAGGCTCCTC(T/C)-3′ (sense), 5′-TT(C/T)CTC(T/G)GCCAGA(A/G)GTGGCCCCCA-3′ (antisense), for HAS3.

To eliminate contamination of the genomic DNA in the PCR template, the extracted total RNA was treated with RNase-free DNase I (Stratagene, La Jolla, CA, USA) for 30 minutes. PCR was performed in a buffer containing 15 mM MgCl2 (Perkin Elmer, Oceanport, NJ, USA) with 0.2 mM deoxyribonucleoside triphosphate (Perkin Elmer), 0.2 µM of each primer, and 0.5% Ampli Taq Gold (Perkin Elmer).

The PCR conditions were set at 1 minute at 94°C (denaturation), 1 minute at 55°C to anneal for HAS1, 1 minute at 58°C for HAS2, 1 minute at 56°C for HAS3, and 1 minute at 72°C (extension). All of these were amplified for 35 cycles. For the negative control, RT-PCR was performed in the absence of the RT step. Amplified products were subcloned into TA vectors (Invitrogen, San Diego, CA, USA) and sequenced at the nucleotide level. The RT-PCR products were electrophoresed in 1% agarose gels in the presence of ethidium bromide, and visualized by ultraviolet fluorescence.

**Growth Factor Treatment and Quantitative RT-PCR**

Subconfluent cultures of HMVECs were cultured for 24 hours in growth factor-free HuMedia-MvG supplemented with 1% fetal bovine serum (v/v). Then, the HMVECs were incubated in HuMedia-MvG containing recombinant human transforming growth factor-β1 (TGF-β1); (10 ng/mL) (R&D Systems, Minneapolis, MN, USA).
and/or recombinant human platelet-derived growth factor-BB (PDGF-BB) (10 ng/mL); (R&D Systems) for 8-48 hours in a humidified atmosphere containing 5% CO₂ at 37°C.

Furthermore, to investigate the effect of TGF-β₁ and PDGF-BB on the regulation of HAS2 expression at the mRNA level, real-time quantitative RT-PCR was performed using the LightCycler technology with a specific fluorescent hybridization probe (Roche Diagnostics GmbH, Mannheim, Germany). Sequences of the primers were as follows: 5′-TCATAAGGCAGCAGCAGGAAG-3′ (sense), 3′-TAATCCAGCTCTTCTACAGGG-5′ (antisense), for HAS2. Sequences of the fluorescent probes were as follows: LC Red 640 (5′-TAAATCCAGCTCTTCTACAGGG-3′), 5′-TAATCCAGCTCTTCTACAGGG-FITC(3′), for HAS2. The probes were designed to hybridize to the target strand so that both dyes were in close proximity. These two probes hybridize during the PCR annealing step. The donor fluorophore (fluorescein) is excited by an external light source, and then passes on part of its excitation energy to the adjacent acceptor (LightCycler Red 640). The excited acceptor fluorophore then emits measurable light. In advance, to examine the optimal conditions of PCR reaction, melting curve analysis was performed with SYBR GreenI hybridization probe. In the LightCycler system, to acquire the fluorescence of a PCR product of unknown concentration (HAS), the fluorescence of several dilutions of subcloned HAS plasmid in pcR2.1 (Invitrogen) was used as an external standard. In order to normalize samples for variations in the amount of cDNA, human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes that are ubiquitously expressed, were used as an internal control. 

In this study, only TGF-β₁ was used. In the pathogenesis of proliferative vitreoretinal diseases, TGF-β₂ is also reported to be involved; however, the effects of TGF-β₁ and TGF-β₂ are not different in the in vitro experiments.²²,²⁸ For this reason, only TGF-β₁ was used in the present study.

**Western Blot Analysis**

HMVECs were incubated in HuMedia-MvG with recombinant human TGF-β₁ (10 ng/mL) and/or recombinant human PDGF-BB (10 ng/mL) for 48 hours. The cellular proteins were then isolated using ISOGEN (Nippon Gene, Toyama), and aliquots were mixed with an equal volume of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 3 minutes at 95°C. The sample buffer contained 100 mM Tris-HCl (pH 6.8), 0.001% bromphenol blue, 20% glycerol, 4% SDS, and 12% mercaptoethanol. Then, the samples were separated on a 10% polyacrylamide gel and electrotransferred to polyvinyliden fluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked for 1 hour at room temperature, followed by incubation with 3.2 μg/mL affinity purified anti-HAS2 polyclonal antibody for 1 hour at room temperature. Following five washes in Tris-buffered saline Tween-20 (TBS-T; DAKO, Carpinteria, CA, USA), the membrane was incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG from donkey; Amersham Pharmacia), which was diluted 25,000-fold in TBS-T. Immune complexes were detected by Enhanced Chemiluminescence (ECL Plus Western blotting reagent pack, Amersham Pharmacia) according to the recommendations of the manufacturer.

**Immunohistochemistry**

Immunohistochemical studies were performed to determine whether HAS1 and HAS2 were expressed in proliferative membranes obtained from the eyes of patients with proliferative diabetic retinopathy (PDR, n = 12), proliferative vitreoretinopathy (PVR, n = 7), and age-related macular degeneration (AMD, n = 4). The expression of HAS3 was not examined because an antibody to HAS3 was not available.

The specimens were obtained during vitreous surgeries after securing informed consent from the patients. This study was approved by the Ethics Committee of Toho University, Sakura Hospital. The clinical characteristics of the subjects are listed in Table 1.

The 6-μm-thick frozen sections were mounted on 3-aminopropyl triethoxysilane-coated slides. The sections were fixed in ice-cold acetone for 10 minutes and then rinsed in phosphate-buffered saline (PBS) solution (0.1 mol/L, pH 7.4). The sections were treated with 3% H₂O₂ in PBS solution for 15 minutes to block the endogenous peroxidase activity, and then rinsed in PBS solution followed by incubation with normal goat serum for 20 minutes at room temperature to block nonspecific binding of the antibodies. Sections were then incubated with the primary antibody (anti-human HAS1 polyclonal antibody, anti-human HAS2 polyclonal antibody) overnight at 4°C in a moist chamber. The binding sites of the primary antibody were detected by goat anti-rabbit immunoglobulins (IgG) conjugated to peroxidase-labeled dextran polymer (EnVision; DAKO, Copenhagen, Denmark). For negative control, the samples were treated with nonimmunized IgG in place of the primary antibody.
### Results

**Expression of HAS Isoforms in HMVECs at the mRNA Level**

RT-PCR, performed on total RNA obtained from HMVECs, showed amplified bands for HAS1, HAS2, and HAS3 at 212 base pairs (bp), 609 bp and 551 bp, respectively (Figure 1). The amplified bands of the cDNA fragments for the three HAS isoforms corresponded to the reported sequences of human HAS1, HAS2, and HAS3 and were identical to the reported sequences (GenBank, U59269 for HAS1, U54804 for HAS2, and U86409 for HAS3). No bands were detected in the negative control. These results confirmed that HAS1, HAS2 and HAS3 are expressed in HMVECs at the mRNA level.

**Effect of Growth Factors on HAS Isoform Expression**

TGF-β and PDGF-BB have been shown to exhibit stimulatory effects on HA synthesis in mesothelial and fibroblast cultures. Among the three HAS isoforms investigated, the HAS2 isoform was most markedly upregulated or downregulated in response to external stimuli. Therefore, by using real-time quantitative RT-PCR, we investigated the effects of TGF-β1 and/or PDGF-BB on the expression level of HAS2 at the mRNA level in HMVEC cultures. Treatment of HMVECs with TGF-β1 or PDGF-BB for 8 hours led to 4- and 2-fold increases of HAS2 transcripts, respectively, over the level of non-stimulated cell cultures (Figure 2; \( P < .009 \), Mann–Whitney U-test). A combination of the two factors has also been investigated; an increase in hyaluronan synthesis to a similar extent as that observed in response to PDGF-BB occurred (Figure 2). Thus, hyaluronan synthesis is influenced differently in response to TGF-β1 and PDGF-BB.

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#### Table 1. Immunohistochemical Expression of HAS1 and HAS2 in Proliferative Tissue

<table>
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<th>HAS2</th>
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*Quantified by counting the average number of cells in three \( \times 1000 \) objective fields = ±: 1–4, +: 5–20, ++: ≥21.
HAS EXPRESSION IN PROLIFERATIVE DISEASES

Effect of Growth Factors on Expression of HAS2 at the Protein Level

The increased expression of HAS2 induced by TGF-β1 (10 ng/mL) was also confirmed at the protein level by Western blot analysis (Figure 3). A protein migrating at approximately 70 kDa was detected in cell lysates obtained from cells treated with TGF-β1 for 48 hours, but not from untreated cells. A considerably weaker band was detected after 24 hours of treatment, suggesting that TGF-β1 induces HAS2 protein accumulation with relatively slow kinetics. The experiments were repeated two times with similar results.

Immunohistochemical Observations of HAS Expression in Proliferative Membranes

To investigate the clinical significance of HAS expression in cells and new vessel formation, experiments were performed to determine whether HAS isoforms were expressed in proliferative membranes. The proliferative membranes obtained from eyes with PDR contained vascular endothelial cells, fibroblast-like cells, other types of cells, and large amounts of ECM (Figure 4A, case 9 in Table 1). HAS1 was expressed in the vascular endothelial cells, and the cells surrounding the new vessels (Figure 4A, right).

The histological features of the proliferative membrane from an eye with PDR (case 7 in Table 1) are shown in Figure 4B. New vessels were not present, and HAS2 was expressed in some of the fibroblast-like cells (spindle-shaped) and in other round-shaped cells. These cells have not been identified but may be macrophage-like cells and/or undifferentiated cells. Some of these cells also expressed HAS1 (Figure 4B, right).

The histological features of the proliferative membrane from an eye with PVR (case 13 in Table 1) are shown in Figure 4C. HAS2 was also expressed in this membrane without new vessel formation but with many fibroblast-like cells and large amounts of ECM (Figure 4C, right).

The expression patterns of the 23 proliferative membranes obtained from eyes of patients with PDR, PVR, or AMD are presented in Table 1. In the membranes from PDR eyes with new vessel formation, HAS1 and HAS2 were detected in the vascular endothelial cells and in fibroblast-like cells (cases 7–12). In some specimens from PDR eyes without new vessels (cases 1–6), HAS1 and HAS2 were not detected probably because very few cells were present in the large amounts of ECM. In the membranes from PVR eyes (cases 13–19), HAS1 and HAS2 were detected in the fibroblast-like cells, macrophage-like cells, and other round-shaped cells. In the subretinal fibrovascular membranes from type 2 AMD eyes (cases 20–23), HAS1 and HAS2 were not detected in smooth muscle cells contained in the membranes.

To determine the activity of proliferative membrane, the density of cells in the sections from each membrane was graded (called “Cellularity” in Table 1). The expression of HAS1 and HAS2 was correlated with this index, i.e., high cellularity or high proliferative activity was correlated with the expression of HAS1 and HAS2.

Discussion

Angiogenesis is a multi-step process and is regulated by various factors, including growth factors, cytokines, and molecules contained in the ECM. HA is found in the ECM and it is involved in angiogenesis by regulating the activity of endothelial cells. HA affects angiogenesis in two ways, depending on its molecular weight; native or high molecular weight HAS does not stimulate

Figure 2. Effects of transforming growth factor-β1 (TGF-β1) and/or platelet-derived growth factor-BB (PDGF-BB) hyaluronan synthase isoform 2 (HAS2) gene expression in human microvascular endothelial cells. Subconfluent cultures were stimulated with 10 ng/mL of TGF-β1, PDGF-BB, or a combination thereof for 8 hours, respectively, and the expression level of mRNA for HAS2 was determined using a real-time quantitative reverse transcription polymerase chain reaction. Mean ± SD of expression levels are shown for each treatment of five separate experiments. *P = .009 (Mann–Whitney U-test).

Figure 3. Western Blot analysis of hyaluronan synthase isoform 2 (HAS2) protein expression. Cell lysates untreated or treated with 10 ng/mL transforming growth factor-β1 for 24 or 48 hours were subjected to sodium dodecyl polyacrylamide gel electrophoresis followed by immunoblotting. The blots were incubated with affinity purified specific antibodies against HAS2.
Figure 4. Immunohistochemical detection of hyaluronan synthase isoform 1 (HAS1) and HAS2. (A) Left: the proliferative membranes obtained from the eye of a patient with proliferative diabetic retinopathy (PDR; case 9 in Table 1). Hematoxylin staining visualizes vascular endothelial cells, fibroblast-like cells and other types of cells as well as extracellular matrix (ECM) molecules. Bar = 50 µm, untreated. Right: Immunohistochemical staining for HAS2 expression, using specific antibodies, revealed that HAS2 is significant in vascular endothelial cells (arrows) and in the cells surrounding the new vessel. Bar = 50 µm. (B) Left: proliferative membrane obtained from the eye of a patient with PDR (case 7 in Table 1). Hematoxylin staining of tissue sections. Bar = 50 µm, untreated. Right: The expression of HAS1 of macrophage-like cells (arrow), undifferentiated cells (arrowheads) and small amount of ECM without new vessel formation can be seen. Bar = 50 µm. (C) Proliferative membrane obtained from the eye of a patient with proliferative vitreoretinopathy (case 13 in Table 1). Left: untreated tissue. Many fibroblast-like cells and large amounts of ECM can be seen. Right: the expression of HAS2 in many fibroblast-like cells (arrows). Bar = 50 µm.
angiogenesis, but does stimulate units of 3–10 disaccharides derived from stimulated angiogenesis. A synergism between the HA degradation products and vascular endothelial growth factor (VEGF) plays an important role in the regulation of angiogenesis, and the oligosaccharides of HA affect intracellular signal transduction and activate cellular functions.

These findings prompted us to investigate the regulation of HA and its relation to regulation of the formation of new vessels and ocular proliferative membranes. HA synthesis is catalyzed by HAS, and HAS expression is regulated by various growth factors and cytokines which, in turn, are related to the pathogenesis of proliferative membranes. Taken together, investigation of HAS expression and its regulation by growth factors may be a new approach to determine the development of proliferative membranes. HAS is important in the pathogenesis of proliferative vitreoretinal diseases. However, there has been no evidence that HAS is expressed in the human vascular endothelial cells. In the present study, we investigated and demonstrate for the first time the expression of HAS in these cells and tissues.

Our study revealed that the induction of HAS2 and protein were markedly stimulated by TGF-β1, whereas PDGF-BB exhibited weaker stimulatory effect. Similar variations have been found previously: in mesothelial cells, PDGF-BB induced an upregulation of HAS2 and a slight induction of HAS1 and HAS3. However, TGF-β1 reduced HAS2 mRNAs slightly. In cultured corneal endothelial and trabecular meshwork cells, both TGF-β1 and PDGF-BB induced HAS2 mRNAs. These studies also demonstrated that the expression is different depending on the cell type and cell confluency.

Studies in the Chinese hamster ovary (CHO) cells transfected with each one of the three HAS isoforms revealed that each HAS isoform may interact specifically with different cytoplasmatic proteins that may have accessory or regulatory roles in hyaluronan biosynthesis and in regulating the size of hyaluronan chains. The effects of HA on vascular endothelial cells may depend on its molecular weight, and this has not been determined in the ocular proliferative membranes.

TGF-β is increased in diabetic retinopathy and regulates angiogenesis through stimulation of HA production. Hypoxia enhances the effects of TGF-β in stimulating HA production. Considered together, these observations support the important roles of TGF-β and HA in the pathogenesis of proliferative diabetic retinopathy.

To investigate whether the HAS expression is related to the proliferative vitreoretinal membrane formation, we examined the expression of HAS1 and HAS2 in the proliferative membranes obtained from eyes of patients with PDR, PVR, or AMD. Both HAS1 and HAS2 were detected in both vascular and nonvascular cells of the PDR membranes and most predominately in nonvascular cells of the PVR membranes. No expression was detected in the membranes from AMD subjects. Because these proliferative membranes are populated with similar cell types, our findings suggest that the regulation of the expression of HAS1 and HAS2 is most likely related to the cellular activities of the component cells and not the cell types. Importantly, HAS expression and activity has been reported to be related to cell proliferation and/or migration. In addition, various growth factors and cytokines stimulate HAS expression.

Taken together, the results of this study have shown that the HAS isoforms are expressed not only in cultured endothelial cells but also in cells populating proliferative membranes obtained from eyes of patients with proliferative vitreoretinal diseases, and thus may be key molecules in the events that control progression of the proliferative diseases.

References