

Expression Regulation of Hyaluronan Synthase in Corneal Endothelial Cells

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PURPOSE. Our previous study showed that hyaluronan synthase (HAS), the enzyme protein of hyaluronan (HA) biosynthesis, is expressed in ocular tissues including the corneal endothelium. In the current study, the mechanism that regulates HAS expression in bovine corneal endothelial cells (BCECs) was investigated.

METHODS. Cultured BCECs were used. HAS expression in BCECs at the mRNA level was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis. The effects of transforming growth factor (TGF)- β and platelet-derived growth factor (PDGF)-BB on HAS expression were examined by quantitative RT-PCR. The involvement of the Smad family (intracellular signal transducer of TGF- β) was also investigated. The expression of HAS in BCECs at the protein level was confirmed by immunocytochemistry and Western blot analysis.

RESULTS. Three HAS isoforms in BCECs were expressed at the mRNA level. The transcriptional sizes of each HAS in BCECs were 4.9 kb for HAS1, 2.8 kb for HAS2, and 1.6 kb for HAS3. The expression of HAS2 at the mRNA level was stimulated by TGF- β 1 and/or PDGF-BB treatment. In contrast, HAS1 and HAS3 expression was not affected by these growth factors. The additive effects of TGF- β 1 and PDGF-BB were observed in the stimulation of the expression levels of HAS2. HAS2 upregulation by these growth factors was also detected by Western blot analysis. The stimulation of the expression of HAS2 at the mRNA level by TGF- β was accelerated by the overexpression of Smad2, Smad3, and Smad4 and inhibited by that of Smad7, all of which were confirmed to be involved in the signal transduction from TGF- β through HAS expression.

CONCLUSIONS. Although three HAS isoforms were expressed in the corneal endothelial cells, the expression of HAS2 was upregulated by TGF- β 1 and/or PDGF-BB. HAS2 expression was regulated by TGF- β through Smad family members. (*Invest Ophthalmol Vis Sci.* 2000;41:3261-3267)

Hyaluronan (HA), a glycosaminoglycan, is composed of β 1,4-linked repeating disaccharides of glucuronic acid β 1,3-linked to *N*-acetylglucosamine.¹ This molecule has several physiochemical and biologic functions such as space filling, lubrication, and providing a hydrated matrix through which cells can migrate.^{2,3} HA is expressed in the ocular tissue as an extracellular matrix (ECM) and plays many roles in its homeostasis and pathophysiology.⁴⁻¹¹

HA is synthesized at the plasma membrane and extruded through a putative membrane pore directly into the extracellular space.¹²⁻¹⁴ The HA synthase (HAS) family contains three isoforms in mammals (HAS1, HAS2, and HAS3) that have been

recently cloned.^{12,15-19} According to previous reports, HA biosynthesis is regulated by four growth factors: transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF)-BB, fibroblast growth factor (FGF), and epidermal growth factor (EGF).²⁰⁻²³ In addition, it has been found that HAS1 and HAS2 expression at the mRNA level is upregulated by TGF- β in human skin cells.²⁴ Although three HAS isoforms show high sequence homology, their chromosomal localizations are different.²⁵ These results suggest that HA synthesis may be regulated by the different expressions of these HAS enzymes by a particular cell type and/or phenotype.²⁶

In our previous study, we showed that all three types of HAS isoforms were expressed at the mRNA level in cultured bovine corneal endothelial cells.²⁷ In addition, the *in situ* corneal endothelial cells expressed HAS1 at the protein level.²⁷ The biologic differences among these isoforms, however, have not yet been resolved. In the present study, we investigated HAS expression regulation to gain further insight into functional differences among HAS isoforms.

MATERIALS AND METHODS

Cell Culture

Bovine corneal endothelial cells (BCECs) of the second passage were used in this study. The cultured cells were prepared according to methods reported previously.^{27,28} A brief sum-

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TABLE 1. Degenerated Oligonucleotide Primers Used in RT-PCR

HAS Family	Degenerate Primer
HAS1	Forward, 5'-TAC TTG GTA GCC TTC AAT GTG GA-3' Reverse, 5'-TAC TTG GTA GCA TA(A/G) CCC AT-3'
HAS2	Forward, 5'-GAA AGG GCC TG(C/T) CAG TCT TAT TT-3' Reverse, 5'-TT(C/G) GT(A/G) AG(A/G) TGC CTG TC(A/G) TCA CC-3'
HAS3	Forward, 5'-GAG CGG GCC TGC CAG TCC TAC TT-3' Reverse, 5'-TTG GT(A/G) AGG TGC CG(A/G) TCA TCC CC-3'

mary of the process is as follows: Eyes were obtained from 3-year-old cattle in an abattoir. Corneal buttons were prepared within 3 hours after enucleation. BCECs with Descemet's membrane were obtained under microscope and separated in the presence of trypsin-EDTA (Gibco, Grand Island, NY). Cells were cultured in 60-mm culture dishes (Falcon, Lincoln Park, NJ) in Eagle's minimum essential medium (EMEM, Gibco) with 15% fetal bovine serum (FBS) and 20 μ g/ml gentamicin (Gibco) under a humidified atmosphere of 5% CO₂ at 37°C. When cells grew to confluence, they were subcultured with trypsin-EDTA treatment.

Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a cDNA template derived from mRNA of BCECs according to methods reported previously.²⁷ Total RNA was isolated from BCECs using a commercially available system (Isogen; Nippon Gene, Toyama, Japan), and the extracted total RNA was treated with RNase-free DNase I (Stratagene, La Jolla, CA) for 30 minutes to avoid genomic DNA contamination. cDNA was made using a reverse transcriptase (Super Script II; Gibco, Grand Island, NY). The degenerated oligonucleotide primers for RT-PCR were based on well-conserved amino acid sequences that were included in the catalytic regions in each human and mouse HAS isoform (Table 1). PCR was performed in 1.5 mM MgCl₂ with 0.2 mM of each dNTP, 0.4 mM of each primer, and 0.025 U/ μ l polymerase (Ampli Taq Gold; Perkin-Elmer, Oceanport, NJ). The PCR sequence used was as follows: 35 cycles of 1 minute at 94°C, 1 minute at 55°C (HAS1), 59°C (HAS2), 57°C (HAS3), 1 minute at 72°C, with an initial 9-minute denaturation step, and a final 7-minute elongation step. The PCR products were visualized on a 1% agarose gel (Sigma, St. Louis, MO). The amplified fragments obtained from RT-PCR were also subcloned into TA vectors (Invitrogen, San Diego, CA) and sequenced according to the manufacturer's protocols for further confirmation of identification.

Northern Blot Analysis

mRNA expression was confirmed by Northern blot analysis using the RT-PCR fragments as hybridization probes. cDNA

restriction fragments encoding complete catalytic regions of HAS1, HAS2, and HAS3 were used as probes after confirmation of the sequences. Using Oligotex-dT30 super (Takara, Otsu, Japan), mRNA was isolated from total RNA that was derived from BCECs using the method described. mRNA (2 μ g/lane) was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nylon filters (Hybond-N⁺; Amersham Pharmacia Biotech, Buckinghamshire, UK). The filters were prehybridized in buffer containing 50% formamide, 5 \times Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 5 \times SSC, and salmon sperm DNA at 42°C and hybridized at 42°C in fresh buffer with each HAS probe. The cDNA probes were labeled with a randomly primed DNA labeling kit using [α -³²P]dCTP (Amersham Pharmacia). Filters were washed twice in 2 \times -0.5 \times SSC, 0.1% SDS for varying times at 42°C. The hybridized and washed filters were exposed to an imaging plate (BAS-MP 2040P; Fujifilm, Tokyo, Japan) and visualized using an image analyzer (BAS 2000; Fujifilm).

Competitive RT-PCR

To observe HAS expression regulation at the mRNA level by TGF- β and/or PDGF-BB, recombinant human TGF- β 1 and/or PDGF-BB (R&D, Minneapolis, MN) at 10 ng/ml were applied to BCECs in EMEM with 1% FBS after starvation in EMEM with 1% FBS for 24 hours. After BCECs were incubated for 12 hours in 5% CO₂ at 37°C, the culture cells were harvested as the sample for mRNA expression determination. The expressions of HAS mRNA were evaluated by a quantitative RT-PCR system (LightCycler; Roche Diagnostics, Mannheim, Germany) with specific fluorescein hybridization probes.^{29,30}

The sequences of the primers and hybridization probes are shown in Table 2. The detection probes were two, independent, single-labeled oligonucleotides that hybridize adjacently on the amplicon internal to the flanking PCR primers. The upstream primer is labeled with a fluorescent (FL) dye at the 3' terminus, and the downstream primer is labeled with a red dye (LightCycler Red 640; Roche) at the 5' end. The sequence of these probes were complementary to the anti-sense strand of each HAS (Table 2). To find the optimum PCR reaction conditions, we tested the melting curve analysis using

TABLE 2. Primers and Hybridization Probes for Competitive RT-PCR

HAS Family	Primer Sets for RT-PCR	Hybridization Probe
HAS1	Forward, 5'-GGCTTGCCAGAGCTACTTTC-3' Reverse, 5'-GCCACGAAGAAGGGGAA-3'	5'-CTTCCGCGAGTGGCTTGTACAA-3' 5'-GCGCTCTGGTGGCACC GGCA-3'
HAS2	Forward, 5'-TGACAGGCATCTAACGAACC-3' Reverse, 5'-TCTAAGGCAGCAGGCAAAAAG-3'	5'-CCCTTCTTTCTCATTGCCAC-3' 5'-TAATCCAGCTCTTCTACAGGG-3'
HAS3	Forward, 5'-GAGTCCTGAGTCTTGCTAC-3' Reverse, 5'-GCCTTGATAATGCCACTA-3'	5'-ACAGGTTTCTTCCCCTTCTTC-3' 5'-TCATTGCCACGGTTCATACAGC-3'

green fluorescent dye (SYBR Green I; Roche) before performing sample quantification.

The 20- μ l PCR reaction mix contained 4 mM (HAS2, HAS3) or 3 mM (HAS1) MgCl₂ with 1.0 mM of each primer, 0.2 mM of FL probe, 0.4 mM of red probe, and 10% volume of master hybridization probes mixture (a LightCycler DNA; Roche). PCR amplification began with a 2-minute denaturation step at 95°C, followed by 50 cycles of 0 seconds at 95°C, 15 seconds at 54°C, and 13 seconds at 72°C. The fluorescein density of the red, reflecting the amount of formed PCR product, was read at the end of each annealing step. To compare expression level of HAS, a dilution series of subcloned HAS plasmid in pCR 2.1 (Invitrogen) was used as internal control and assayed in each RT-PCR run. These values were entered on the quantitative RT-PCR system's input screen as concentration standards. Relative quantification of the HAS signals was performed by normalizing the HAS signals with glucose-3-phosphate dehydrogenase (G3PDH) by PCR cloning. Thus, G3PDH mRNA amounts were determined to normalize the sample cDNA content. Other controls included in each run were water blanks and samples without the reverse transcription step.

The quantification procedure was as follows: A standard curve was made for the target gene (HAS) and housekeeping gene (*G3PDH*) using the dilution series of the target or G3PDH plasmid. The log concentrations of the target (X) and the housekeeping gene were calculated (Y) from a standard curve. The expression levels were then standardized and compared (X/Y) between each sample. Thus, standardizing log concentration values (X/Y) should provide the relative amount of HAS mRNA. The statistic analysis was performed by Mann-Whitney test.

Transfection with cDNA of the Smad Family by Electroporation

To observe the involvement of the Smad family in signal transduction to regulate HAS mRNA expression, the transfection of the cDNA of the Smad family was performed by electroporation. These Smad cDNA constructs, which were subcloned in pcDNA 3.1 (Invitrogen), were generously provided by Kohei Miyazono and Takeshi Imamura of The Cancer Institute (Japanese Foundation for Cancer Research, Tokyo, Japan).²⁸ The electrode was 60 mm in diameter with 4 mm gap electrodes manufactured by T-R-Tech (Tokyo, Japan). Electroporation was performed in phosphate-buffered saline (PBS, pH 7.4) containing cDNA at a final concentration of 10 μ g/ml. Cells were exposed to 10 pulses at 60 V for 96 msec with an electroporator (CUY 21; T-R-Tech). The tissue culture plates were then incubated in EMEM with 5% FBS at 37°C in 5% CO₂. In the preliminary study, approximately 50% to 70% of the cells were transfected with plasmids for the expression of green fluorescent protein (GFP; Clontech, Palo Alto, CA) using the method described, and electroporation did not significantly affect cell viability (data not shown).

After transient transfection of cDNA of Smad2, Smad3, Smad4, or Smad7 (the concentration of each Smad cDNA was 10 μ g/ml) into BCECs by electroporation, the cells were incubated for 12 hours in serum-free medium (Opti MEM; Gibco). Cells were then incubated in EMEM with 1% FBS in the presence or absence of 10 ng/ml TGF- β 1 and/or PDGF-BB for 12 hours. Sample cDNA for quantitative RT-PCR was then obtained as has been described.

Immunocytochemistry

The expression of HAS2 at the protein level in BCECs was detected immunocytochemically. The cells were washed with PBS twice and fixed with 70% ethanol for 15 minutes. After they were washed and fixed, the samples were treated with nonimmunized goat serum for 20 minutes at room temperature to avoid nonspecific binding of the antibodies. They were then incubated with the affinity-purified anti-human HAS2 polyclonal antibody (a generous gift from a coauthor [PH]) as the primary antibody at 10 μ g/ml at room temperature for 1 hour.^{31,32} For the negative control, nonimmunized serum IgG (Vector, Burlingame, CA) was used in place of the primary antibody. Immunoreactivity was detected with a kit (Histofine SAB-PO; Nichirei, Tokyo, Japan), according to the manufacturer's protocol. In brief, the cells were incubated with biotinylated anti-rabbit goat serum for 15 minutes at room temperature and then rinsed with PBS, after which they were incubated with a streptavidin-biotin-peroxidase complex for 10 minutes at room temperature. The final reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB).

Western Blot Analysis

Recombinant human TGF- β 1 and/or PDGF-BB (R&D) was applied to BCECs in EMEM with 1% FBS after starvation in EMEM with 1% FBS for 24 hours. After BCECs were incubated for 24 hours in 5% CO₂ at 37°C, medium was removed and the cells washed in PBS. The cell proteins were obtained by using an isolation kit (Isogen; Nippon Gene), according to the manufacturer's protocol. In brief, cell proteins were isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol. Proteins were then washed in 0.3 M guanidine hydrochloride in 95% ethanol (three times) and were solubilized by 1% SDS. The same volume of the sample buffer was added to the protein solution, and the sample was boiled for 3 minutes. The sample buffer contained 100 mM Tris-HCl (pH 6.8), 0.001% bromophenol blue, 20% glycerol, 4% SDS, and 12% β -mercaptoethanol. The concentration of protein sample was measured by spectrophotometer to adjust the amount of total protein among samples. The samples were applied to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted onto polyvinylidene fluoride membranes (Amersham Pharmacia). For the primary antibody, Anti-HAS1 and HAS2 polyclonal antibodies (generous gifts from a coauthor [PH]) were used.^{31,32} Binding was visualized by a Western blot analysis reagent pack and detection reagents (ECL Plus; Amersham Pharmacia).

RESULTS

Expression of the HAS Family in BCECs at the mRNA Level

RT-PCR showed the amplified bands by each set of the primers for HAS1, HAS2, and HAS3. The sizes were 533 bp, 609 bp, and 585 bp, respectively (data not shown). The amplified cDNA fragments were sequenced to identify the HAS isoforms. As shown in Figure 1, the species homology in the catalytic region of each HAS isoform was well conserved. The PCR fragments were aligned to human or mice HAS1, HAS2, and HAS3 with the highest identity of 100%, 98.9%, 99.4% at the amino acid level, respectively. (The accession numbers of the nucleotide

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HUM HAS1 No.293-RACQSYFHCVSCISGPLGLYRNNLLQQFLEAWYNQKFLGTHCTFGDDRHLNRMLSMGY
BOV HAS1 .....
MOU HAS1 .....
HUM HAS2 .....G..Q.....M...S..HE.V.D...E.M.NQ.S.....V..L..
BOV HAS2 -----G..Q.....M...S..HE.V.D...E.M.SQ.S.....V..L..
MOU HAS2 .....G..Q.....M...S..HE.V.D...E.M.NQ.S.....V..L..
HUM HAS3 ----..G..Q.....M...S.....D..H.....SK.S.....V..L..
BOV HAS3 -----D..H.....SK.S.....V..L..
MOU HAS3 .....G..Q.....M...S.....D..H.....SK.S.....V..L..

HUM HAS1      ATKYTSRRCYSETPSSFLRWLSQQTRWSKSYFREWLYNALWWHRHHAMTYEAVVSGLF
BOV HAS1 .....
MOU HAS1 .....
HUM HAS2 .....A..K.LT...IEY...N.....M.F.K..L.....IIT.F.
BOV HAS2 .....A..K.LT...IEY...N.....M.F.K..L.....IT.F.
MOU HAS2 .....A..K.LT...IEY...N.....M.F.K..L.....IT.F.
HUM HAS3 R...A..K.LT...TKY...N.....S..F.K..L.....S..T.F.
BOV HAS3 R...A..K.LT...TKY...N.....S..F.K..L.....S..T.F.
MOU HAS3 R...A..K.LT...TRY...N.....S..F.K..L.....S..T.F.

HUM HAS1      PFFVAATVLRFLFYAGRPWALLWVLLCVQGVALAKAAFAAWLRGCLRMVLLSLYAPLYMCG
BOV HAS1 -----
MOU HAS1 .....
HUM HAS2 ...LI...IQ...R.KI.NI.LF..T..L.G.I.SS..SC...NIV..FM...SV...SS
MOU HAS2 ...LI...IQ...R.KI.NI.LF..T..L.G.I.SS..SC...NIV..FM...SV...SS
HUM HAS3 ...LI...IQ...R..I.NI.LF..T..L.G.II..TY.CF...NAE..IFM...SL...SS
BOV HAS3 ...LI...IQ...R..I.NI.LF..T..L.G.II..TY.CF...NAE..IFM...SL...SS
MOU HAS3 ...LI...IQ...R..I.NI.LF..T..L.G.II..TY.CF...NAE..IFM...SL...SS

HUM HAS1      LLPAKFLALVTMNQSG-No.487
BOV HAS1 -----
MOU HAS1 .....
HUM HAS2 .....MF.IA.I.KA.
BOV HAS2 .....MF.IA.I.KA.
MOU HAS2 .....MF.IA.I.KA.
HUM HAS3 .....IF.IA.I.K.-
BOV HAS3 .....IF.IA.I.K..
MOU HAS3 .....IF.IA.I.K..

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FIGURE 1. Amino acid sequence alignment of vertebrate HAS enzyme. Amino acid sequences at the catalytic regions of bovine HAS were obtained from degenerate RT-PCR products. These sequences were aligned with reference to human HAS1 (hHAS1) amino acids 293 to 487. Dots indicate the same amino acid residue as hHAS1. Sequence differences among vertebrate HAS enzymes were conserved among species. h, human; m, mouse; b, bovine.

sequences reported in this article are AB017803 for HAS1, AB017804 for HAS2, and AB017805 for HAS3 of GenBank/EMBL/DOBJ). Because the similarities of sequences at the nucleotide level were low among bovine HAS isoforms, these fragments were used as probes in Northern blot analysis.

mRNA expression of the three HAS isoforms in BCECs was confirmed by Northern blot analysis at the baseline. Three HAS isoforms were hybridized in 4.9 kb for HAS1, 2.8 kb for HAS2, and 1.6 kb for HAS3 (Fig. 2). The data shown in Figure 2 were derived from the experiments using the same filter after rehybridization. The detected sizes of mRNA were different among HAS isoforms, which also confirms that the RT-PCR fragments did not cross react for the detection of HAS isoforms.

Regulation of Expression of HAS in BCECs at the mRNA Level

Recently, a new on-line fluorescein PCR detection system has been introduced^{29,30} (LightCycler, Roche). Originally, the so-called HybProbe chemistry was developed to be used in the system. Figure 3 shows the effects of TGF- β and/or PDGF-BB in the expression of HAS2. TGF- β or PDGF-BB stimulated HAS2 expression at the mRNA level (Fig. 3). Additive effects of TGF- β and PDGF-BB were also observed in HAS2 production (Fig. 3). The expression of HAS1 and HAS3 was lower than that of HAS2 in BCECs, and the upregulation effects by TGF- β and/or PDGF-BB were not detected in HAS1 or HAS3 synthesis (data not shown).

HAS2 expression at the mRNA level was accelerated by the overexpression of Smad2, Smad3, or Smad4 in case of TGF- β stimulation, and inhibited by that of Smad7 (Fig. 4). Otherwise, HAS2 mRNA was not accelerated by the overexpression of Smad families in the presence of PDGF-BB or in the absent of TGF- β (Fig. 4 shows only the case of Smad4). The additive effects of TGF- β and PDGF-BB were not observed in Smad transfected cells (Fig. 4 shows only the case of Smad4).

Expression and Regulation of HAS in BCECs at the Protein Level

The cultured BCECs were stained positively in plasma membrane and partial cytoplasm by anti-HAS2 antibody (Fig. 5). These results show that BCECs express HAS2 at the protein level. To observe the effects of TGF- β and/or PDGF-BB to HAS2 expression at the protein level, Western blot analysis was performed. As shown in Figure 6, HAS2 expression in BCECs was upregulated by TGF- β and/or PDGF-BB at the protein level. Molecular mass of HAS2 was approximately 70 kDa. Figure 6 shows the results of Western blot analysis repeated three times.

DISCUSSION

We previously reported the partial sequences of bovine HAS isoforms derived from BCECs.²⁷ In the current study, to gain

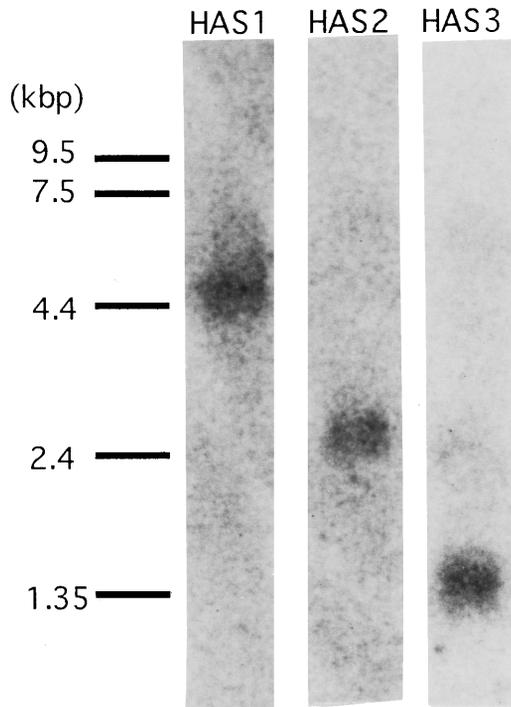


FIGURE 2. Bovine HAS gene expression in BCECs (Northern blot analysis). Radiolabeled probes were sequentially hybridized to Northern blot analysis of mRNA from BCECs. Filters were exposed to an imaging plate to be used in a bioimaging analyzer. Expression of all three HAS isoforms in BCECs was confirmed. These isoforms were hybridized in 4.9 kb for HAS1, 2.8 kb for HAS2, and 1.6 kb for HAS3.

detailed catalytic regions of the bovine HAS sequence, longer fragments of HAS isoforms were obtained by RT-PCR and sequenced after TA cloning (Fig. 1). We found great sequence similarities at the nucleic acid level and at the amino acid level among species homologues of the HAS family (Fig. 1). These

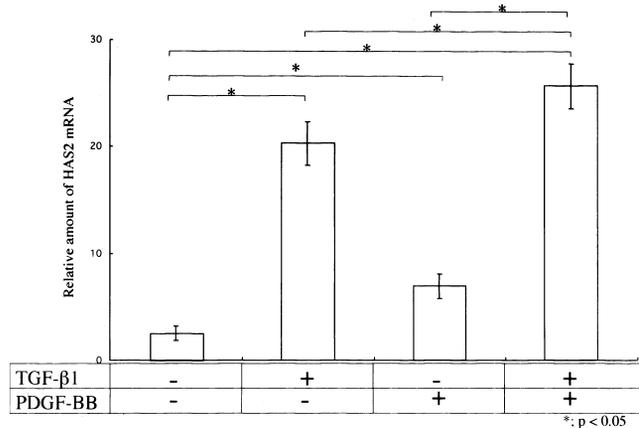


FIGURE 3. Quantitative determination of HAS2 mRNA by TGF-β1 and/or PDGF-BB. HAS2 expression at mRNA level by TGF-β1 and/or PDGF-BB in BCECs was analyzed by competitive PCR. Real-time fluorescein PCR detection was used in this experiment ($n = 6$, error bar is SD). The y -axis shows standardized log concentration values that are obtained from a standard curve and shows the relative amount of HAS2 mRNA. HAS2 mRNA in BCECs was accelerated by TGF-β1 and/or PDGF-BB (10 ng/ml).

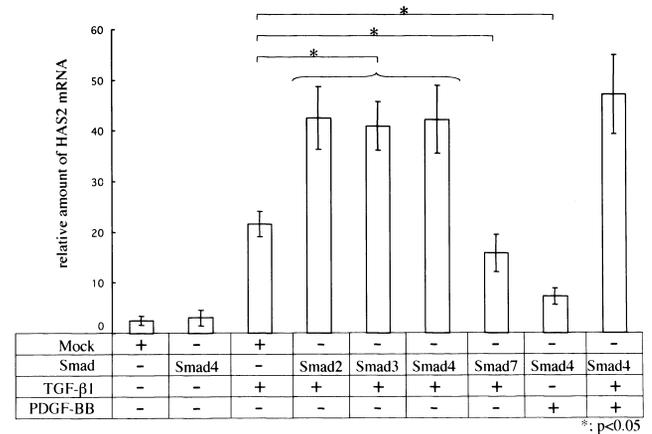


FIGURE 4. Involvement of Smad family in HAS2 production regulation. To investigate the involvement of Smad family members in HAS2 production regulation by TGF-β, Smad cDNA (10 μg/ml) was transfected into BCECs using electroporation. Real-time fluorescein PCR detection was used in this experiment ($n = 6$, error bar is SD). The y -axis is as in Figure 3 and shows the relative amount of HAS2 mRNA. HAS2 mRNA was accelerated by overexpression of Smad2, Smad3, and Smad4 in the presence of TGF-β1 and inhibited by that of Smad7 (each concentration of TGF-β1 was 10 ng/ml). Overexpression of Smad family members did not affect HAS2 production in the presence of PDGF-BB and in the absence of TGF-β. Additive effects of TGF-β and PDGF-BB were not observed in transfected cells.

results showed that the catalytic regions of the HAS sequence were well conserved among vertebrate species and were compatible with previous reports.^{12,25}

The expression of HAS at the mRNA level in BCECs was confirmed by Northern blot analysis using the probes obtained from RT-PCR fragments (Fig. 2). Results obtained from human tissue blotting, HAS1 was the most widely expressed with the major transcript of 4.6 kb and a smaller transcript of 2.4 kb.²⁵ HAS2 transcripts were detected to be 4.4 kb and 3.2 kb,²⁵ and HAS3 transcripts were hybridized in 4.3, 3.0, and 2.4 kb.²⁵ These cells, however, were not derived from ocular tissues. This is the first report of transcription sizes of HAS that are derived from bovine ocular tissue. The differences in transcription size may be due to species and tissue differences.

Little has been known about the functional characterization of HAS isoforms. Spicer and McDonald²⁵ reported that HAS2 and HAS3 were involved in the formation of HA-depend-

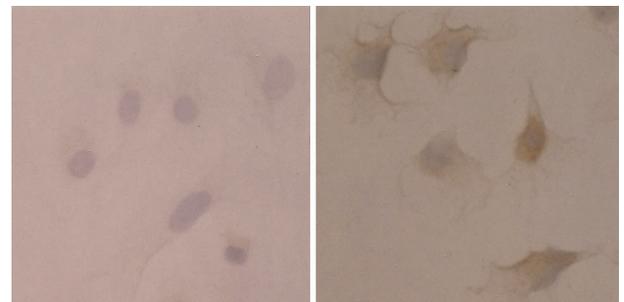


FIGURE 5. Expression of HAS in BCECs at the protein level. BCECs after treatment with nonimmunized serum IgG (negative control, left) and anti-HAS2 polyclonal antibody (right). BCECs are stained positively in plasma membrane and partial cytoplasm with anti-HAS2 antibodies.

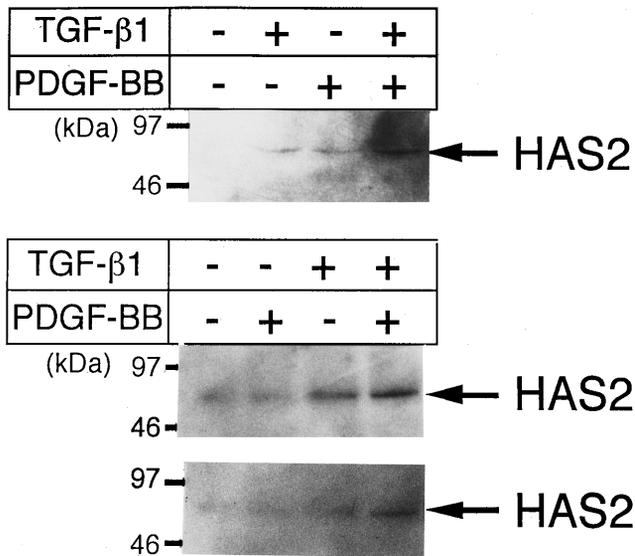


FIGURE 6. Effect by TGF- β and/or PDGF-BB in HAS2 expression at the protein level (Western blot analysis). After starvation in EMEM with 1% FBS for 24 hours, 10 ng/ml TGF- β 1 and/or PDGF-BB was added to BCECs in EMEM with 1% FBS. Cells were incubated for 24 hours. Western blot analysis was performed using affinity-purified anti HAS2 polyclonal antibody. TGF- β 1 and/or PDGF-BB accelerate HAS2 production. In each lane, total protein concentration of sample was adjusted to the same by spectrophotometer. This figure shows the results of Western blot analysis repeated three times. Those loaded samples were extracted from different cultured cells.

dent pericellular coats in cultured cells. The latest study by Itano et al.³³ showed distinct enzymatic properties among HAS isoforms. As the first step to investigate the differences in biologic functions among HAS isoforms in corneal endothelial cells, we examined the expression regulation of the HAS enzymes using a new online fluorescein quantitative PCR with fluorescein hybridization probes in the present study. This capillary PCR system produces quantitative results extremely rapidly by using a newly developed heating and cooling technique.²⁹ The fluorescein-based format for PCR detection was the hybridization probe format. These two single-labeled hybridization probes are nonextendable. After excitation by the LED, a fluorescence resonance energy transfer (FRET) occurs from the FL to the red dye (LC-Red 640; Roche), increasing the dye's signal, which is directly related to the amount of PCR product before the plateau is reached. FRET is only effective when energy donor and acceptor molecules are in proximity (within 1 to 5 bp). This condition is fulfilled in our hybridization probes, which are shown in Table 2.

It is well known that ECM production is regulated by several growth factors.²⁹ TGF- β and PDGF-BB stimulate HA production.²⁰⁻²³ In human cultured dermal cells, the expressions of both HAS1 and HAS2 mRNA were accelerated by TGF- β .²⁴ In this study, only HAS2 expression at the mRNA and protein level was stimulated by TGF- β and/or PDGF-BB, which did not promote HAS1 or HAS3 expression (Figs. 3, 6). These results may suggest the differences in potential roles among HAS isoforms. We have speculated that in the corneal endothelium, HAS2 expression is increased in cases of biologic dynamic states including wound healing and ocular inflammation; however, HAS1 and HAS3 are expressed in a constitutive manner, not in an inducible manner.

It is interesting that the additive effect of TGF- β and PDGF-BB was observed in HAS2 production (Figs. 3, 6). This observation would suggest TGF- β 1 signal cross talk downstream to that of PDGF-BB in the intracellular and/or transcriptional level. Because the cross talk mechanism between TGF- β 1 and PDGF-BB is still unclear, unknown cytokine networks involved in ECM production regulation should be investigated in the future projects.

Because TGF- β elicited HAS2 expression at the mRNA level (Fig. 3), the signal transduction pathways in HAS2 from TGF- β was investigated. We transfected cDNA of Smad family members (intracellular signal transducers of TGF- β ^{34,35}) to BCECs and observed HAS2 expression quantitatively using a competitive RT-PCR assay. HAS2 production at the mRNA level was accelerated by the overexpression of Smad2, Smad3, or Smad4 in the presence of TGF- β 1 and inhibited by that of Smad7 (Fig. 4). This result is very similar to laminin production at the protein level, which was reported previously.²⁸

The overexpression of Smad family in the presence of PDGF-BB did not affect the HAS mRNA production (Fig. 4). Furthermore, the additive effects by TGF- β 1 and PDGF-BB were not observed by the overexpression of Smad family members (Fig. 4). There are two possibilities to explain these results: There is no cross talk between TGF- β 1 and PDGF-BB at Smad levels, and the activity of HAS production stimulation was saturated by the transfection of Smad cDNA. We must investigate the transcriptional regulation mechanism of HAS isoforms, for which the analysis of promoter regions of each gene is mandatory.

The present study is the first achievement of HAS expression regulation in ocular cells. It is necessary in future studies to investigate more carefully the functional features of HAS isoforms and cytokine network mechanisms.

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