Hyaluronan Synthase Expression in Bovine Eyes

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Purpose. Hyaluronan (HA), a high-molecular-weight linear glycosaminoglycan, is a component of the extracellular matrix (ECM). It is expressed in eyes and plays important roles in many biologic processes, including cell migration, proliferation, and differentiation. Hyaluronan is produced by HA synthase (HAS), which has three isoforms: HAS1, HAS2, and HAS3. In this study, the HAS expression in the anterior segment of bovine eyes was investigated to determine the significance of HA in eyes.

Methods. To obtain bovine HAS probes, degenerate oligonucleotide primers, based on well-conserved amino acid sequences including the catalytic region of each HAS isoform, were used for reverse transcription-polymerase chain reaction to amplify mRNA from bovine corneal endothelial cells (BCECs). Hyaluronan synthase-1 expression in the anterior segment of bovine eyes at the protein level was investigated by immunohistochemistry.

Results. All three HAS isoforms were expressed in BCECs at the mRNA level. Amplified cDNA fragments of HAS1, HAS2, and HAS3 from BCECs can be aligned to human counterparts, showing similarities of 100%, 97.3%, and 100%, respectively, at the amino acid level. Hyaluronan synthase 1 was expressed at the protein level in corneal epithelium, keratocyte, corneal endothelium, conjunctival epithelium, ciliary epithelium, capillary endothelium, and trabecular meshwork.

Conclusions. Hyaluronan synthase isoforms were expressed in the ocular anterior segment and are speculated to be involved in HA production in situ.

Hyaluronan (HA), a high-molecular-weight linear glycosaminoglycan, is a component of the extracellular matrix (ECM). It is expressed in eyes and plays important roles in many biologic processes, including cell migration, proliferation, and differentiation. Hyaluronan is produced by HA synthase (HAS), which has three isoforms: HAS1, HAS2, and HAS3. In this study, the HAS expression in the anterior segment of bovine eyes was investigated to determine the significance of HA in eyes.

Materials and Methods

Cell Culture

Second-passage bovine corneal endothelial cells (BCECs) were selected for use in this study. The cultured cells were prepared according to the methods reported previously.16,17 Briefly, eyes were obtained from 3-year-old cattle at an abattoir. Corneal buttons were prepared within 3 hours after enucleation. BCECs with Descemet’s membrane were collected under a 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 and 20 μg/ml gentamicin (Gibco) in a humidified atmosphere with 5% CO2 at 37°C. When the cells grew to confluence, they were subcultured into a 10-cm culture plate (Falcon) treated with trypsin-EDTA.

Reverse Transcription–Polymerase Chain Reaction

In the cultured BCECs, the expression of the HAS family at the mRNA level was investigated using reverse transcription–polymerase chain reaction (RT-PCR), which was performed using the cDNA template derived from mRNA of BCECs. Total RNA was isolated from BCECs using Isogen® (Nippon Gene, Tokyo, 113-8655 Japan).
Toyama, Japan), and cDNA was produced using reverse transcriptase (Super Script II; Gibco). The degenerate oligonucleotide primers for RT-PCR were designed based on well-conserved amino acid sequences of human and mouse HAS isoforms, which include the catalytic region in each human HAS isoform.¹⁴ Table 1. The sense primer for HAS1 is located in exon 3, and the antisense primer is located in exon 4, according to the genomic structure of mouse HAS1.¹⁵ Both primers for HAS2 and HAS3 are located in exon 4, according to the genomic structure of mouse HAS2 and HAS3.¹⁵ The genomic structure of the species homologies are conserved within the genes, which have been cloned, and therefore, the genomic structures for the bovine HAS family genes are speculated to be similar.¹⁶ To eliminate the possibility of the contamination of genomic DNA in the PCR template, the extracted total RNA was treated with RNase-free DNase I (Stratagene, La Jolla, CA) for 30 minutes. PCR was performed in buffer containing 15 mM MgCl₂ (Perkin Elmer, Oceanport, NJ) with 0.2 mM of each deoxyribonucleoside triphosphate (Perkin Elmer), 0.2 µM of each primer, and 0.5% AmpliTaq Gold (Perkin Elmer). The conditions for PCR were as follows: 30 cycles of 1 minute at 94°C, 1 minute at 57°C, and 1 minute at 72°C, with an initial 9-minute denaturing step and a final 5-minute elongation step. For the negative control, RT-PCR was performed in the absence of the reverse transcriptase step. The fragments amplified by RT-PCR were subcloned into TA vectors (Invitrogen, San Diego, CA), sequenced at the nucleotide level and deduced to an amino acid sequence.

### Table 1. Degenerate Primer Sequences and Amino Acid Sequences of the HAS Family

<table>
<thead>
<tr>
<th>HAS Family</th>
<th>Degenerate Primer</th>
<th>Corresponding Amino Acid Sequence of Human HAS</th>
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<tr>
<td>HAS1</td>
<td>Forward: 5'-TAC TGT GTA GCA TTC AAT GTG GA-3'</td>
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<td>Reverse: 5'-TAC TGT GTA GCA TAC(A/G) CCC AT-3'</td>
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<td>Reverse: 5'-TT(C/G) GTG(A/G) AG(A/G) TGC CTG TCA(A/G) TCA CC-3'</td>
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<td>Reverse: 5'-TTG GT(A/G) AGG TGC CG(A/G) TCA TCC CC-3'</td>
<td>No. 516—GDDRHLTN—No. 323</td>
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Results

Expression of the Hyaluronan Synthase Family in BCECs at the mRNA Level

RT-PCR showed amplified bands of the expected sizes by using the degenerate primer sets for HAS1, HAS2, and HAS3 (Fig. 1). The bands were detected even after DNase I treatment, which eliminated the possibility that the amplified bands were derived from genomic DNA (Fig. 1). When RT-PCR was also performed in the absence of the reversed transcriptase step to eliminate the possibility of contamination, the amplified band was not detected (data not shown). The amplified cDNA fragments were sequenced at nucleotide levels, and amino acid sequences were determined (Fig. 2). The fragment using the HAS1 degenerate primer set was best aligned to human and mouse HAS1 with the highest identity of 100% at the amino acid level, as was the fragment of bovine HAS1 (Figs. 2, 3). Similarly, the fragments produced by using the HAS2 or HAS3 degenerate primer sets corresponds to that of bovine HAS2 or

**Immunohistochemistry**

Only the antibody of HAS1 was available in our research group.¹⁸ The expression of HAS1 in the anterior segment of the bovine eyes were detected immunocytochemically. Frozen sections of the bovine anterior segment of the eye were each cut 8-µm thick and placed on poly-L-lysine-coated slides. The frozen sections were washed twice in phosphate-buffered saline (PBS) and fixed in cold 4% paraformaldehyde in 0.1 M PBS for 5 minutes. After washing and fixation, the sections were treated with nonimmunized goat serum for 30 minutes at room temperature to avoid nonspecific binding of the antibodies and were incubated with the affinity-purified anti-human HAS1 polyclonal antibody as the primary antibody at 10 µg/ml at room temperature for 1 hour.¹⁸ For the negative control, PBS was used in place of the primary antibody. Immunoreactivity was detected with a commercial kit (Histofine SAB-PO; Nichirei, Tokyo, Japan), according to the manufacturer’s protocol. In brief, the sections were incubated with biotinylated anti-rabbit goat serum for 15 minutes at room temperature and then rinsed in 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.
HAS3 (Figs. 2, 3). Only the above three isoform fragments were obtained from BCECs by RT-PCR under various conditions. (The nucleotide and amino acid sequences reported in this article have been submitted to GenBank/EMBL/DDBJ. The accession numbers are AB017803 for HAS1, AB017804 for HAS2, and AB017805 for HAS3.)

Expression of HAS1 at the Protein Level

In the anterior segment of bovine eyes, HAS1 expression was detected in the corneal epithelium, keratocyte, corneal endothelium, conjunctival epithelium, vascular endothelium and smooth muscle, nonpigmented epithelium of the ciliary body, and trabecular meshwork in comparison with expression in the negative control specimens (Fig. 4).

DISCUSSION

The results of the present study show that all three HAS isoforms are expressed in bovine cultured corneal endothelial cells at the mRNA level (Fig. 1). According to previous reports, the HAS gene family consists of three members in humans and mice.14,15 There is a difference in the distribution among the HAS family15: In humans, HAS1 and HAS3 are widely expressed, but HAS2 is prevalent in the heart and submucosa of the small intestine.15 The present study is the first to show that corneal endothelial cells express all three isoforms of HAS at the mRNA level. The functional differences between the three HAS isoforms were also reported; HAS2 and HAS3 were involved in the formation of the coat around the cultured cells containing HA, in which HAS1 was not involved.15 These HAS isoforms in the corneal endothelium may also play different roles, for example, in wound healing processes, including cell extension and migration. In future studies, it is mandatory to clarify the functional differences among the HAS isoforms and the molecular mechanisms of regulation in corneal endothelial cells.

Rittig et al.19,20 reported that primate and monkey eyes expressed HAS in corneal endothelium, corneal epithelium, conjunctival epithelium, nonpigmented epithelium, stromal fibroblast, capillary endothelium, and trabecular meshwork detected by using a polyclonal antibody to Streptococcus pneumoniae. By using affinity-purified antihuman HAS1 polyclonal antibody in our study, we confirmed that HAS1 is expressed at the protein level in the anterior segment of bovine eyes. Corneal endothelium, ciliary epithelium, and trabecular meshwork expressing HAS1 suggests that HAS in these tissues produces HA by HAS in aqueous humor. Hyaluronan is also distributed in the trabecular meshwork of normal eyes.9,10 The expression of HAS1 in trabecular meshwork suggests that HA may originate from cells of the trabecular meshwork. The other possibility is that HA in the trabecular meshwork may originate from aqueous humor. Knepper et al.10 reported that HA content is decreased in trabecular meshwork in primary open-angle glaucoma in comparison with that in trabecular meshwork in normal subjects. Thus, HAS function may be related to the pathogenesis of glaucoma. Tears also contain HA,15 which suggests that HAS in corneal and conjunctival epithelia are involved in the production of HA in tears by HAS.

This is the first report to show that HAS isoforms are expressed at the mRNA level and at the protein level in the anterior segment of bovine eyes. In our study HA expressed in eyes was produced by ocular tissue through HAS functions. In future studies, the functional significance of the HAS isoforms in eyes should be investigated.

<table>
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FIGURE 3. Similarity in HAS family (amino acid level). Fragments that were produced using HAS degenerate primers were aligned to human or mouse HAS1, HAS2, and HAS3 with highest identity of 100%, 97.3%, 100% (bold type) at the amino acid level, respectively.
FIGURE 4. Expression of HAS1 in the anterior segment of the bovine eye after treatment with PBS (negative control, left column) or anti-HAS1 polyclonal antibody (right column). (A, B) cornea; (C) conjunctiva and sclera; (D) ciliary process; (E) vascular endothelium and smooth muscle in ciliary body stroma; (F) trabecular meshwork (angle). Corneal epithelium (A), keratocyte (B, arrows), corneal endothelium (B, arrowheads), conjunctival epithelium (C), vascular endothelium (C, E), vascular smooth muscle (E), nonpigmented epithelium of ciliary body (D, arrowheads), and trabecular meshwork (F, arrow) expressed HAS1. Original magnification, (A, C, F) ×260; (B) ×3500; (D, E) ×650.

References


ANNOUNCEMENT

Glaucome Research Foundation Seeks International Ophthalmology Fellows

The Glaucoma Research Foundation (GRF), a U.S.-based non-profit organization, seeks applicants for a one-year program providing ophthalmologists from developing countries with research training opportunities in the United States. GRF places up to three ophthalmologists in U.S. research centers where at least two-thirds of their time is in clinical or laboratory research. Candidates should be assistant professors with a strong interest in glaucoma research. Candidates must be fluent in written and spoken English. Candidates must return to home countries to advance glaucoma research and patient treatment.

Candidates must be nominated by department chairs, complete an application form, forward curriculum vitae and letter confirming academic position. Visit http://www.glaucoma.org/fellowship.html or contact GRF to request an application form.

Deadline is July 15, 1999 for fellowship beginning July 1, 2000.

GRF, 200 Pine Street, Suite 200, San Francisco, CA 94104, USA. Phone (415) 986-3162, FAX (415) 986-3763.