

# Constitutive Expression of Inter- $\alpha$ -inhibitor (I $\alpha$ I) Family Proteins and Tumor Necrosis Factor-stimulated Gene-6 (TSG-6) by Human Amniotic Membrane Epithelial and Stromal Cells Supporting Formation of the Heavy Chain-Hyaluronan (HC-HA) Complex\*

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**Background:** The heavy chain-hyaluronan (HC-HA) complex from amniotic membrane (AM) has anti-inflammatory, anti-scarring, and anti-angiogenic actions.

**Results:** AM epithelial and stromal cells constitutively expressed I $\alpha$ I and TSG-6 supporting the production of HC-HA.

**Conclusion:** AM is an extrahepatic tissue constitutively producing its own I $\alpha$ I and HC-HA complex.

**Significance:** HC-HA endogenously produced by AM may play an important protective role during fetal development.

Recently, we reported HC-HA, a covalent complex formed between heavy chains (HCs) of inter- $\alpha$ -inhibitor (I $\alpha$ I) and hyaluronan (HA) by the catalytic action of tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6), is responsible for human amniotic membrane (AM) anti-inflammatory, anti-scarring, and anti-angiogenic actions. At the present time, the only well characterized source of I $\alpha$ I is serum being produced by the liver. This study showed that AM epithelial and stromal cells and stromal matrix all stained positively for HA, HC 1, 2, and 3, bikunin, and TSG-6. TSG-6 mRNA and protein were constitutively expressed by cultured AM epithelial and stromal cells without being up-regulated by TNF. In serum-free conditions, these cells expressed I $\alpha$ I, leading to the formation of HC-HA complex that contained both HC1 and HC2. In contrast, only HC1 was found in the HC-HA complex purified from AM. Local production of I $\alpha$ I, the HC-TSG-6 intermediate complex, and HC-HA were abolished when cells were treated with siRNA to HC1, HC2, bikunin (all of which impair the biosynthesis of I $\alpha$ I), or TSG-6 but not to HC3. Collectively, these results indicate that AM is another tissue in addition to the liver to constitutively produce I $\alpha$ I and that the HC-HA complex made by this tissue is different from that found at inflammatory sites (e.g. in asthma and arthritis) and in the matrix of the cumulus oocyte complex.

The human inter- $\alpha$ -inhibitor (I $\alpha$ I)<sup>3</sup> family is a group of plasma serine protease inhibitors that comprise one or two homologous heavy chains (HCs) and one common light chain called bikunin, of which the latter is responsible for the protease inhibitory activity (1, 2). The homologous HCs consist of five members (HC1–5), with HC1, HC3, and HC4 residing in a closely linked region on chromosome 3p21 (3), whereas HC2 and HC5 are arranged in tandem on chromosome 10p15 (4). HC1, HC2, and HC3 contain a conserved proteolytic cleavage site in their C-terminal regions and are able to link to the single chondroitin sulfate (CS) chain of the proteoglycan bikunin via covalent (ester) bonds after they are released from their HC precursor (1, 2, 5, 6). HC4 lacks such a conserved sequence in its C-terminal region, explaining why it does not attach to bikunin and exists as a free HC polypeptide in the blood circulation (7). HC5 apparently has all of the structural features of HC1–3 necessary for the covalent attachment to CS; however, the existence of a HC5-bikunin complex has not been proven (4). The bikunin gene encodes bikunin and another serum protein,  $\alpha_1$ -microglobulin, which is separated from bikunin after coupling with HCs.

The I $\alpha$ I family proteins are primarily synthesized and assembled in the liver and secreted into the blood circulation. In human plasma, I $\alpha$ I (composed of HC1, HC2, and bikunin) is present at a concentration of 0.15–0.5 mg/ml together with pre- $\alpha$ -inhibitor (P $\alpha$ I, composed of HC3 and bikunin), which has a third of concentration of I $\alpha$ I, and HC2/bikunin (composed of HC2 and bikunin) (8–10). Although the liver is the main site of post-translational modification of the I $\alpha$ I family proteins, expression of individual HCs and bikunin proteins has

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<sup>3</sup> The abbreviations used are: I $\alpha$ I, inter- $\alpha$ -inhibitor; AM, amniotic membrane; AMEC, AM epithelial cell; AMSC, AM stromal cell; CS, chondroitin sulfate; HA, hyaluronan; HAase, hyaluronidase; HABP, HA-binding protein; HC, heavy chain; P $\alpha$ I, pre- $\alpha$ -inhibitor; sc, scrambled; SHEM, supplemental hormonal epithelial medium; TSG-6, TNF-stimulated gene-6.

## I $\alpha$ I, TSG-6, and HC-HA Expression by Amniotic Membrane Cells

been detected in many other tissues such as lung, kidney, spleen, pancreas, testis, brain, colon, cartilage, and placenta (4, 9, 11–13). During the acute phase of inflammation or stress, the hepatic synthesis of bikunin and HC2 genes is down-regulated, that of HC3 and HC4 genes is up-regulated, whereas that of HC1 gene is not changed (14–16). Thus, bikunin, HC2/bikunin, and I $\alpha$ I molecules are viewed as negative acute phase proteins whereas P $\alpha$ I is a positive one, implying the relevance of the I $\alpha$ I family proteins in inflammation.

Hyaluronan (HA), a high molecular mass ( $2.0 \times 10^5$  to  $1.0 \times 10^7$  Da) nonsulfated glycosaminoglycan, consists of linear repeating disaccharide units of glucuronosyl-*N*-acetylglucosamine and distributes ubiquitously in the extracellular matrix of vertebrate tissues. Unlike sulfated glycosaminoglycans, HA is not covalently linked to a core protein but does interact noncovalently with a wide range of HA-binding proteins, including aggrecan, cartilage link protein, CD44, and tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6) (17). Intriguingly, HC1 and HC2 of I $\alpha$ I as well as HC3 of P $\alpha$ I can form covalent (ester) bonds with HA to yield an HC-HA complex (18–20) by the catalytic action of TSG-6 that transfers HCs from I $\alpha$ I and P $\alpha$ I onto HA (19, 21–23). In most cell types, the expression of TSG-6 is not constitutive but stimulated by pro-inflammatory cytokines, e.g. IL-1 and TNF (24–26).

The HC-HA complex is the principal structural component of the cumulus matrix vital for successful ovulation and fertilization, e.g. in the mouse (27, 28). Both the bikunin-null mouse (unable to biosynthesize intact I $\alpha$ I) and the TSG-6-null mouse (unable to transfer HCs from I $\alpha$ I to HA) exhibit female infertility (21, 27, 28). Although HC-HA complexes are also found at sites of inflammation (11, 12, 29), whether they have a pro- or anti-inflammatory role (or both) remains unclear (30, 31). Recently, we purified a HC-HA complex from human amniotic membrane (AM) and demonstrated that it exhibits anti-inflammatory and anti-scarring (32) and anti-angiogenic (33) effects, explaining why clinical transplantation of cryopreserved AM for ocular surface reconstruction exerts these therapeutic actions (34–37). Given the avascular nature of the AM, we speculate that the AM HC-HA complex may be formed by AM cells using I $\alpha$ I locally produced but not derived from the serum.

### EXPERIMENTAL PROCEDURES

**Materials**—Guanidine hydrochloride, cesium chloride, EDTA, anhydrous alcohol, potassium acetate, sodium acetate, sodium chloride, sodium hydroxide, Tris, Triton X-100, 3-(*N,N*-dimethyl palmityl ammonio) propanesulfonate (Zwittergent<sup>3–16</sup>), protease inhibitor mixture (including 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, E-64, leupeptin, and pepstatin A) and phenylmethanesulfonyl fluoride were obtained from Sigma-Aldrich. *Streptomyces* hyaluronidase (HAase), chondroitinase ABC, and biotinylated HA-binding protein (HABP) were from Seikagaku Biobusiness Corporation (Tokyo, Japan). DMEM, Ham's F12 nutrient mixture, FBS, Hanks' balanced salt solution, gentamicin, amphotericin B, and radioimmunoprecipitation assay buffer were purchased from Invitrogen. Slide-A-Lyzer dialysis cassettes (3.5K MWCO) were from Fisher Scientific. The BCA protein assay

kit was from Pierce. The HA Quantitative Test kit was from Corgenix (Westminster, CO). 4–15% gradient acrylamide ready gels and nitrocellulose membranes were from Bio-Rad. I $\alpha$ I and urinary trypsin inhibitor (*i.e.* bikunin) were prepared in our laboratory from human plasma and urine, respectively, according to the published method (1, 38). Recombinant human TNF and human/mouse TSG-6 mAb (MAB2104) were from R&D Systems (Minneapolis, MN). Mouse anti-human ITIH1 polyclonal antibody against full-length ITIH1 and rabbit anti-human bikunin polyclonal antibody against full-length bikunin were from Abcam (Cambridge, MA). The recombinant human TSG-6 protein (TSG-6Q) (39) and rabbit antisera against the C-terminal peptide of human TSG-6 (RAH-1, TSTGNKNFLAGRFSHL), the N-terminal peptides of human HC2 (SLPGESEEMM) and HC3 (SLPEGVANGI), and the C-terminal peptide of human HC2 (ESTPPPHVMRVE) were as described previously (11, 12, 39, 40). PepMute<sup>TM</sup> siRNA Transfection Reagent was from SignaGen Laboratories (Rockville, MD). The RNeasy Mini RNA isolation kit, small interfering RNA (siRNA) oligonucleotides for targeting endogenous human HC1 (UAAUGUUCUGAGGAGUCACCTT) and HC3 (UUGACUAUCUGCAGGUUGCCA), and nontargeting siRNA control oligonucleotides (scrambled RNA) were from Qiagen (Valencia, CA). siRNA oligonucleotide for targeting endogenous human TSG-6 (GGUUUCCAAAUCAAAUAUGUUGCAA), HC2 (GGAUCAAAUAGAGAGCGUUAUCACG), and bikunin (GGUAUUUCUAUAAUGGUACAUCAT) were from OriGene Technologies (Rockville, MD). Western Lighting<sup>TM</sup> Chemiluminescence Reagent was from PerkinElmer Life Sciences. The ultracentrifuge (LM8 model, SW41 rotor) was from Beckman Coulter.

**Cell Cultures**—Human tissue was handled according to the Declaration of Helsinki. The fresh human placenta was obtained from healthy mothers after elective cesarean deliveries in the Baptist Hospital (Miami, FL) via an approval (Protocol 03-028) by the Baptist Health South Florida Institutional Review Board. Primary human AM epithelial and stromal cells (designated as AMECs and AMSCs, respectively) were isolated from fresh placenta as described previously (41, 42) and cultured in supplemental hormonal epithelial medium (SHEM, which consisted of DMEM/F12 (1:1, v/v), 5% (v/v) FBS, 0.5% (v/v) dimethyl sulfoxide, 2 ng/ml EGF, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 5 ng/ml sodium selenite, 0.5  $\mu$ g/ml hydrocortisone, 0.1 nM cholera toxin, 50  $\mu$ g/ml gentamicin, 1.25  $\mu$ g/ml amphotericin B) (41, 43) under a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The culture medium was changed every 2 days. At subconfluence, cells were incubated in SHEM containing 20 ng/ml TNF for 4 or 24 h prior to RT-PCR or Western blot analysis. In experiments for TSG-6 detection, AMECs, AMSCs, and human skin fibroblasts were cultured in DMEM/F12 containing 10% FBS medium (*i.e.* to prevent the influence of other components such as EGF in SHEM on TSG-6 expression). To exclude serum I $\alpha$ I, serum-free cultures were established with secondary cultures. After seeding and attachment, cells were washed three times with Hanks' balanced salt solution and switched to fresh SHEM without serum, and the serum-free medium was changed every 2 days until experimental manipulation.

**TABLE 1**  
PCR primers

Protein	Sense/antisense	Primer sequence	Production size
HC1	Sense	5'-CCACCCCATCGGTTTGAAGTGCT-3'	138
	Antisense	5'-TGCCACGGGTCTTCTGTAGTCT-3'	
HC2	Sense	5'-ATGAAAAGACTCACGTGCTTTTTC-3'	127
	Antisense	5'-ATTTGCCTGGGGCCAGT-3'	
HC3	Sense	5'-TGAGGAGGTGGCCAACCCACT-3'	318
	Antisense	5'-CGTCTTCCAGCAGCTGCTC-3'	
Bikunin	Sense	5'-GTCCGGAGGGCTGTGCTACC-3'	294
	Antisense	5'-GATGAAGGCTCGGCAGGGGC-3'	
TSG-6	Sense	5'-CCAGGCTCCCAAATGAGTA-3'	284
	Antisense	5'-TTGATTTGAAACCTCCAGC-3'	
GAPDH	Sense	5'-ACCACAGTCCATGCCATCAC-3'	452
	Antisense	5'-TCCACCACCTGTTGCTGTA-3'	

*siRNA Transfection*—AMECs and AMSCs were cultured in serum-free SHEM in 6-well plates until 50–60% confluence, when cells were transfected with PepMute™ siRNA Transfection Reagent with or without 10 nM HC1 siRNA, HC2 siRNA, bikunin siRNA, HC3 siRNA, or scrambled (sc) RNA. After 48 h, cells were harvested and subjected to RT-PCR and Western blot analysis. For TSG-6 detection, AMECs and AMSCs were cultured in DMEM/F12 containing 10% FBS medium and transfected with TSG-6 siRNA or scRNA.

*Purification of HC-HA Complex from AM and Serum-free Cultures by Ultracentrifugation*—HC-HA complex was purified from AM and serum-free cultures as described previously (32, 44, 45). For purification of HC-HA complex from AM, cryopreserved human AM, obtained from Bio-tissue (Miami, FL), was sliced into small pieces, frozen in liquid nitrogen, and ground to fine powder by a BioPulverizer. The powder was mixed with cold PBS at 1:1 (g/ml). The mixture was kept at 4 °C for 1 h with gentle stirring and then centrifuged at 48,000 × g for 30 min at 4 °C. The supernatant (designated as AM extract) was then mixed with a 8 M guanidine HCl/PBS solution (at 1:1 ratio of v/v) containing 10 mM EDTA, 10 mM aminocaproic acid, 10 mM N-ethylmaleimide, and 2 mM PMSF (45). For purification of HC-HA complex from serum-free cultures, cells were washed three times with Hanks' balanced salt solution and extracted with 6 M guanidine HCl, 0.2 M Tris-HCl (pH 8.0), 0.1% (w/v) Zwittergent<sup>3-16</sup> containing protein inhibitors (10 mM EDTA, 10 mM aminocaproic acid, 10 mM N-ethylmaleimide, and 2 mM PMSF) (44). The cell extract was kept at 4 °C overnight with gentle stirring before removing the insoluble materials by centrifuging at 14,000 × g for 30 min at 4 °C. The above extracts were adjusted to a density of 1.35 g/ml (AM extract) or 1.40 g/ml (cell extract) with cesium chloride, respectively, and subjected to isopycnic centrifugation at 35,000 rpm, 15 °C, for 48 h. The resultant density gradients were fractionated into 12 tubes (1 ml/tube), in which the contents of HA and proteins were measured using HA Quantitative Test kit and BCA protein assay kit, respectively. Fractions from the first ultracentrifugation, which contained most HA, were pooled, brought to a density of 1.40 g/ml (AM extract) or 1.45 g/ml (cell extract) by addition of CsCl, ultracentrifuged, and fractionated in the same manner as described above. Fractions from the second ultracentrifugation, which contained HA but no detectable proteins, were pooled and dialyzed in distilled water and then precipitated twice with 3

volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate at 0 °C for 1 h. After centrifugation at 15,000 × g, the pellet was briefly dried by air, stored at –80 °C, and designated as AM HC-HA complex and cell HC-HA complex, respectively.

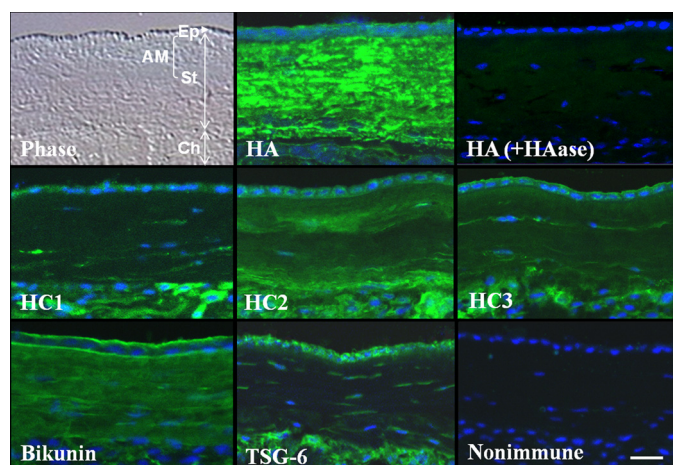
*Immunofluorescence Analysis*—Human fetal membrane containing AM and chorion was cryosectioned to 5-μm thickness, fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 20 min. After blocking with 0.2% (w/v) BSA in PBS for 1 h, sections were incubated with biotinylated HABP (for HA, 5 μg/ml) or different primary antibodies specific for HC1, HC2, HC3, bikunin, and TSG-6 (all diluted 1:200 in blocking solution) overnight in a humidity chamber at 4 °C. For HC2, we used an anti-N-terminal HC2 antibody throughout unless mentioned otherwise. For TSG-6, we used MAB2104 throughout unless mentioned otherwise. After washing with PBS, they were incubated with Alexa Fluor 488 streptavidin (for HA, diluted 1:100), or respective secondary antibodies (*i.e.* FITC-conjugated anti-mouse IgG, or FITC-conjugated anti-rabbit IgG) for 1 h at room temperature. Isotype-matched nonspecific IgG antibodies were used as a control. Alternatively, sections were treated with 50 units/ml *Streptomyces* HAase at 37 °C for 4 h before fixation. Nuclei were stained by Hoechst 33342, and images were obtained using a Zeiss LSM700 confocal laser scanning microscope (Zeiss, Germany).

*RT-PCR*—Total RNA was extracted from AM tissue and cell cultures using a RNeasy Mini RNA isolation kit. The cDNA was reverse-transcribed from 1 μg of total RNA using a Cloned AMW First-Strand cDNA synthesis kit with gene-specific antisense primer (for HC1–3 and bikunin) (Table 1) or oligo(dT) primer (for TSG-6). First-strand cDNAs were amplified by PCR using AmpliTaq Gold Fast PCR Master Mix and the specific gene primers (Table 1) (46–48). Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used to normalize the amounts of the amplified products. The PCR products were electrophoresed on a 2% (w/v) agarose gel with ethidium bromide staining, photographed using the UVP BioImaging system, and analyzed using the ImageJ software (Java).

*Western Blotting*—Culture supernatants were collected, and cell lysates were obtained by washing cells six times with cold PBS followed by incubating in radioimmunoprecipitation assay buffer at 4 °C for 1 h with gentle stirring and centrifugation at

## I $\alpha$ I, TSG-6, and HC-HA Expression by Amniotic Membrane Cells

14,000  $\times$  g for 30 min at 4 °C. Protein concentrations in culture supernatants and cell lysates were quantified with a BCA protein assay kit. For alkaline treatment of AM extract, samples were incubated in 50 mM NaOH for 1 h at 25 °C. For HAase digestion of the HC-HA complex, samples were dissolved in 0.1 M sodium acetate buffer (pH 6.0) and incubated at 60 °C for 1 h with or without 20 units/ml *Streptomyces* HAase. The above samples were resolved by SDS-PAGE on 4–15% (w/v) gradient acrylamide ready gels under denaturing and reducing conditions and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% (w/v) fat-free milk in 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 0.05% (v/v) Tween 20 followed by sequential incubation with different primary antibodies followed by their respective HRP-conjugated secondary antibodies. Immunoreactive proteins were visualized by Western Lighting<sup>TM</sup> Chemiluminescence reagent.

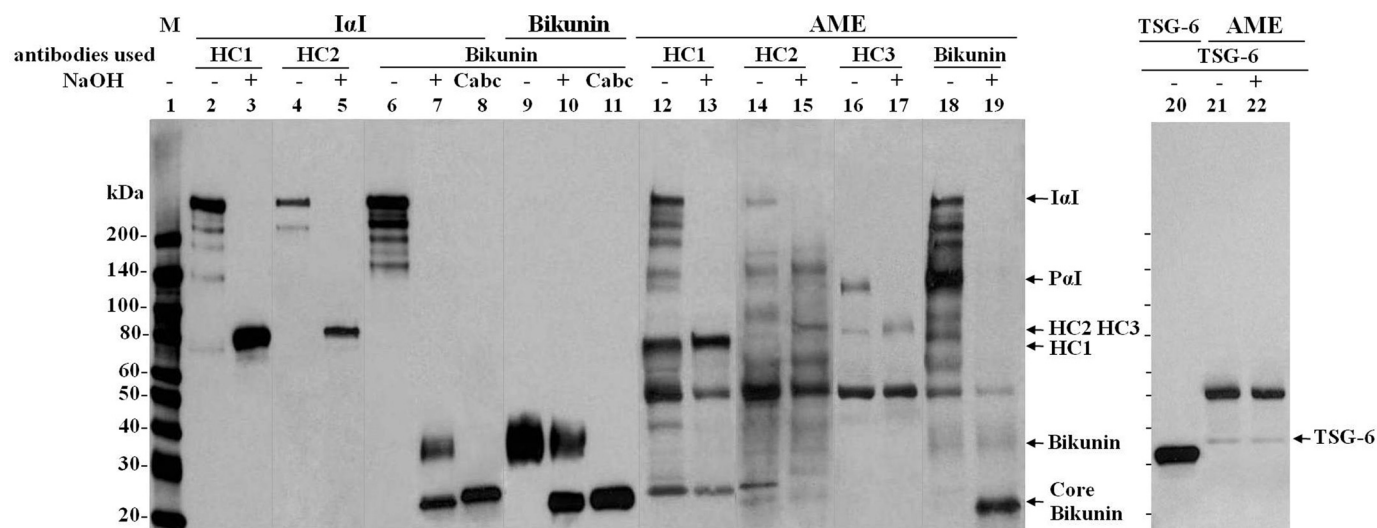


**FIGURE 1. Immunolocalization of HA, HCs, bikunin, and TSG-6 in human AM.** Frozen sections of human AM were probed with biotinylated HABP with or without HAase digestion and with chain-specific antibodies against I $\alpha$ I and P $\alpha$ I components as indicated. Nonimmune rabbit serum was used as a control. Nuclei were counterstained with Hoechst 33342 (blue). Ep, epithelium; St, stroma; Ch, chorion. Scale bar, 25  $\mu$ m.

## RESULTS

**Immunolocalization of HA, HCs, Bikunin, and TSG-6 in Human AM**—To address whether the AM could produce its own I $\alpha$ I to form HC-HA complex, we first investigated whether the required components, *i.e.* HA, each individual HC, bikunin, and TSG-6, were actually present in human AM *in vivo*. Frozen sections of the fetal membrane revealed AM consisting of a simple epithelium and an avascular stroma and subjacent cell-rich chorion (Fig. 1, *Phase*). Consistent with what has been reported previously (49, 50), strong positive HA immunostaining was noted in AM stroma and relatively weak staining in AM epithelium using a biotinylated HABP (Fig. 1, *HA*). This staining was lost when the tissue section was predigested by HAase (Fig. 1, *HA(+HAase)*) indicating that HA staining is specific. Immunostaining of each individual HC using specific antibodies also revealed a positive staining in AM epithelium, stromal cells, and/or stromal matrix (Fig. 1, *HC1*, *HC2*, and *HC3*). Positive bikunin immunostaining was found in the apical surface of the epithelium, the basement membrane zone, and the stroma (Fig. 1, *Bikunin*). TSG-6 immunostaining with two different anti-TSG-6 antibodies, *i.e.* MAB2104 (Fig. 1, *TSG-6*) and RAH-1 (data not shown) showed the same pattern with positive staining associated with AMECs, AMSCs, and stromal matrix. The lack of immunoreactivity by nonimmune control serum indicates that the staining described above is specific. Collectively, these results suggested the presence of all components in AM required for forming I $\alpha$ I, P $\alpha$ I, and HC-HA.

**Presence of Individual HCs, Bikunin, TSG-6, P $\alpha$ I, and I $\alpha$ I in AM-soluble Extract**—To investigate further the presence of the above components in AM, we performed Western blotting analyses of proteins extracted by an isotonic salt buffer before and after 50 mM NaOH treatment to cleave ester bonds (20, 32, 44, 51). Using anti-HC1, HC2, or bikunin antibodies, purified I $\alpha$ I was found to contain a major 250-kDa species corresponding to intact I $\alpha$ I and several weak species of smaller molecular mass most likely representing intermediate species (Fig. 2, *lanes 2, 4, and 6*), including a free HC1 species of 75 kDa (*lane 2*).



**FIGURE 2. Presence of I $\alpha$ I, P $\alpha$ I, individual HCs, bikunin, and TSG-6 in AM extract.** Purified I $\alpha$ I, urinary trypsin inhibitor (*i.e.* bikunin), TSG-6, and AM extract (AME) were treated with or without 50 mM NaOH at 25 °C for 1 h or chondroitinase ABC (*Cabc*) at 37 °C for 2 h before Western blotting using antibodies as indicated. Individual HC1, HC2, HC3, bikunin, and TSG-6 species were found in AM extract. M, protein ladder markers.

NaOH treatment of the I $\alpha$ I preparation released HC1 (75 kDa, lane 3), HC2 (80 kDa, lane 5), and bikunin as 35-kDa and 22-kDa species (lane 7). The latter two species likely correspond to bikunin with and without an attached CS chain (11, 52). When purified I $\alpha$ I was treated with chondroitinase ABC lyase, only the 22-kDa species was observed with the anti-bikunin antibody (lane 8) (53, 54). Purified bikunin, which appeared as a 35-kDa species (lane 9), yielded both 35-kDa and 22-kDa species after the same NaOH treatment (lane 10), but gave rise to only the 22-kDa species after chondroitinase ABC treatment (lane 11). These results confirmed that both 35-kDa and 22-kDa bikunin species formed after mild NaOH treatment of I $\alpha$ I (*i.e.* with partial release of the CS chain). Based on the profile generated by both I $\alpha$ I and bikunin controls, we detected the 250-kDa I $\alpha$ I species and its components, HC1, HC2, and bikunin in AM-soluble extract (lanes 12, 14, and 18). The anti-bikunin antibody also reacted with a 130-kDa species (lane 18), which was likely P $\alpha$ I because it was detected by an anti-HC3 antibody that also recognized a free HC3 species of 80 kDa (lane 16). The identity of I $\alpha$ I and P $\alpha$ I was further verified by the NaOH treatment, which released corresponding HCs and bikunin species that were detected by the various chain-specific antibodies (lanes 13, 15, 17, and 19). A similar result was also obtained with an anti-I $\alpha$ I antibody (data not shown). Because I $\alpha$ I/P $\alpha$ I chain-specific antibodies and the anti-I $\alpha$ I antibody all reacted with a 50-kDa species, which was also detected by normal mouse or rabbit serum (data not shown), we concluded that this 50-kDa species was nonspecific.

Analysis of the AM extract with an anti-TSG-6 antibody (MAB2104) revealed a species of  $\sim$ 35 kDa (lane 21), which corresponds to the expected size of native TSG-6 (25), which is slightly larger than the size of recombinant TSG-6 (TSG-6Q, 32 kDa) (lane 20) (39) that has a lower level of glycosylation (32). This antibody also detected a major 50-kDa species, where neither this nor the  $\sim$ 35-kDa species was affected by NaOH treatment (*i.e.* in agreement with our previous report (32) using three different anti-TSG-6 antibodies). Again, the detection of the 50-kDa species is likely nonspecific. Taken together, these results demonstrated that the soluble AM extract indeed contained I $\alpha$ I, P $\alpha$ I, HC1, HC2, HC3, bikunin, and TSG-6.

**Constitutive Expression of HC1, HC2, HC3, and Bikunin mRNA and Proteins by AMECs and AMSCs in Serum-containing Media**—To provide data on the cellular sources of HC1, HC2, HC3, and bikunin, we established primary cultures of AMECs and AMSCs in SHEM, which was found to be the optimal medium in our prior study (41), and extracted total RNA for RT-PCR and proteins for Western blot analysis. The positive control of human liver RNA yielded PCR products with the expected sizes of 138 bp (HC1), 127 bp (HC2), 318 bp (HC3), and 294 bp (bikunin) (Fig. 3A). These four RT-PCR products were all present in AM tissue as well as both AMECs and AMSCs. The expression of HCs and bikunin transcripts was not greatly up-regulated by TNF in AMECs and AMSCs. Western blotting of AMEC and AMSC lysates showed that a 265-kDa and a 200-kDa species were recognized by the chain-specific antibodies against HC1, HC2, and bikunin (Fig. 3, B, C, and D) but not by anti-HC3 (Fig. 3E), suggesting that these two

species were I $\alpha$ I-related. Anti-HC1, anti-HC2, and anti-HC3 antibodies all recognized  $\sim$ 75-kDa species (Fig. 3, B, C, and E); the anti-bikunin antibody detected a 35-kDa species (Fig. 3D). Thus, based on our comparison with purified I $\alpha$ I and a serum control (as a source of P $\alpha$ I), and with published data (52, 55), AMECs and AMSCs can be concluded to express HC1, HC2, HC3, and bikunin proteins. In addition, both anti-HC1 (Fig. 3B) and anti-HC2 antibodies (Fig. 3C) also recognized 120-kDa species that are likely HC1-TSG-6 and HC2-TSG-6 complexes, respectively (see below) (18, 23, 56). Based on prior reports (52, 55), the 100-kDa species revealed by anti-HC1, anti-HC2, and anti-HC3 antibodies were likely HC1, HC2, and HC3 precursors, respectively. Approximately 45–90-kDa species revealed by anti-bikunin are likely to be bikunin precursor (*i.e.*  $\alpha_1$ -microglobulin/bikunin tandem protein) with or without glycosaminoglycan attached, a finding also observed in primary rat hepatocytes (54). The identities of HC1- and HC2-positive species at 65 and 50 kDa (Fig. 3, B and C) and the faint HC3-positive species at 50 kDa (Fig. 3E) are not clear because these species were also present in serum-free AMEC and AMSC lysates (see below); they were likely not derived from serum. Interestingly, the intensities of the various HC and I $\alpha$ I species were not notably affected by addition of TNF. Overall, these results indicate that AMEC and AMSC produce individual HC1, HC2, and bikunin chains that are assembled into I $\alpha$ I proteins.

**Production of I $\alpha$ I Family Proteins in Serum-free AMEC and AMSC Cultures**—To avoid undue influence by serum I $\alpha$ I and to provide further evidence for the cellular production of I $\alpha$ I family proteins by AMECs and AMSCs, we harvested cell lysates from their respective serum-free cultures. We also treated these two serum-free cultures with HC1 siRNA, HC2 siRNA, and bikunin siRNA (*i.e.* because HC1, HC2, and bikunin are components of I $\alpha$ I); as a comparison, we also treated cells with HC3 siRNA because HC3 is not a part of I $\alpha$ I. RT-PCR analysis confirmed the efficiency of these siRNAs to down-regulate their respective transcripts in these two cultures (Fig. 4A). Western blot analysis showed that the 265-kDa and 200-kDa species were significantly reduced by HC1 siRNA, HC2 siRNA, and bikunin siRNA (Fig. 4B), but not by HC3 siRNA (Fig. 4C). The production of HC1, HC2, and bikunin (and their putative precursors) was notably down-regulated by their respective siRNA (Fig. 4B). In addition, the 120-kDa species recognized by anti-HC1 and anti-HC2 antibodies that likely corresponded to respective HC1-TSG-6 and HC2-TSG-6 complexes was decreased by HC1 siRNA and HC2 siRNA, respectively (Fig. 4B). The HC3 siRNA decreased the HC3 species but did not affect the aforementioned species (Fig. 4C), consistent with the inhibitory effects of the siRNAs against HC1, HC2, and bikunin, shown in Fig. 4B, being specific. Essentially the same results were obtained using AMSCs (data not shown). These results collectively provided further evidence that AMECs and AMSCs produced HC1, HC2, and bikunin proteins, which assemble to form I $\alpha$ I.

**Constitutive Expression of TSG-6 mRNA and Protein by AMECs and AMSCs**—Previous studies have shown that adult skin fibroblasts and peripheral blood mononuclear cells (24, 25, 57, 58), myeloid dendritic cells (59, 60), renal tubular epithelial

## I $\alpha$ I, TSG-6, and HC-HA Expression by Amniotic Membrane Cells

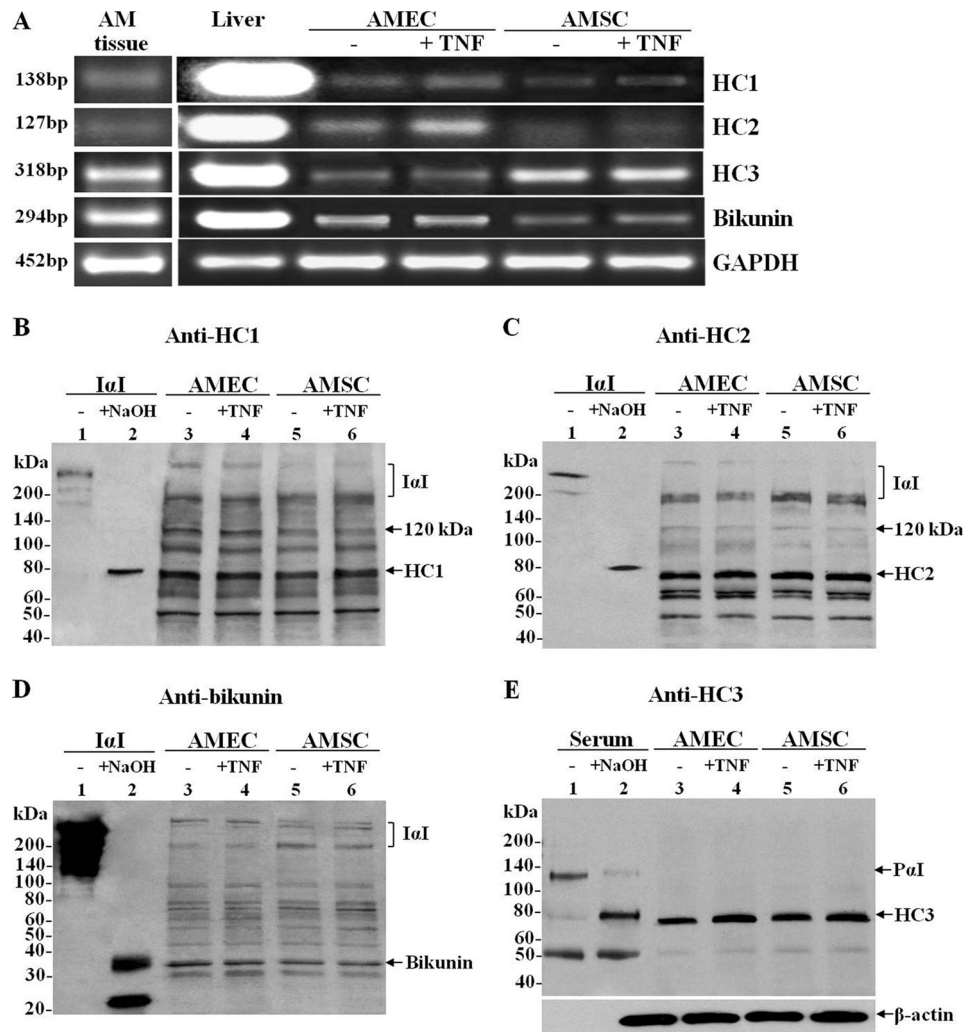


FIGURE 3. **Constitutive expression of HC1, HC2, HC3, and bikunin mRNA and proteins by AMECs and AMSCs.** RNA and protein were extracted from AM tissue and both AMECs and AMSCs cultured in SHEM with or without 20 ng/ml TNF for 4 h (for RT-PCR) or 24 h (for Western blotting). Expression of HC1, HC2, HC3, and bikunin transcripts was compared with liver total RNA using GAPDH as the loading control (A), whereas that of HC1, HC2, HC3, and bikunin proteins was compared with control I $\alpha$ I and serum using  $\beta$ -actin as the loading control (B, C, D, and E, respectively).

cells (61), articular chondrocytes (25, 62, 63), as well as cervical smooth muscle cells (40) express TSG-6 mRNA and protein only under the stimulation of pro-inflammatory cytokine such as TNF and IL-1. To provide further evidence for the cellular sources of TSG-6, we cultured AMECs and AMSCs in DMEM/F12 with 10% FBS to prevent the influence of other components such as EGF in SHEM on TSG-6 expression. As expected, expression of TSG-6 mRNA by human skin fibroblasts was negative but significantly up-regulated by 20 ng/ml TNF (Fig. 5A). In contrast, there was constitutive expression of TSG-6 mRNA by AMECs and AMSCs without being affected by TNF (Fig. 5A). TSG-6 mRNA was also detected in RNAs extracted from fresh AM tissue (Fig. 5A). Western blot analyses of lysates detected four species, *i.e.* 35, 50, 100, and 120 kDa, in both AMECs and AMSCs, but only 35 kDa and two faint species of 50 and 100 kDa in skin fibroblasts (Fig. 5B). Addition of TNF did not change any species in AMECs and AMSCs, but up-regulated the 35-kDa species (but not the 50- or 100-kDa species) in skin fibroblasts (Fig. 5B). In supernatants, we detected 35-, 100-, 120-, and 150-kDa species (but not 50 kDa) in skin fibro-

blasts (Fig. 5C); after TNF stimulation, the 100- and 150-kDa species did not change, but 35- and 120-kDa species became intensified. All of these species were also detected in AMEC and AMSC supernatants, where 35- and 120-kDa species were unaffected by TNF.

TSG-6 siRNA transfection greatly reduced both 35- and 120-kDa species in both lysates and supernatants of AMEC but did not affect the 50- and 100-kDa species in cell lysates (Fig. 5D) or the 100- and 150-kDa species in supernatants (Fig. 5E). The same result was observed in AMSCs (data not shown). From these experiments we concluded that the 35-kDa species corresponded to TSG-6 secreted by AMECs and AMSCs, where its production was induced by TNF in skin fibroblasts but was constitutive in AMECs and AMSCs. On the basis of earlier reports (18, 23, 56), the 120-kDa species likely corresponded to the covalent complexes of TSG-6 with HCs. Because the 50-, 100-, and 150-kDa species were not affected by TSG-6 siRNA and because the amounts of these species were not altered by TNF in skin fibroblasts, they were concluded to be nonspecific.

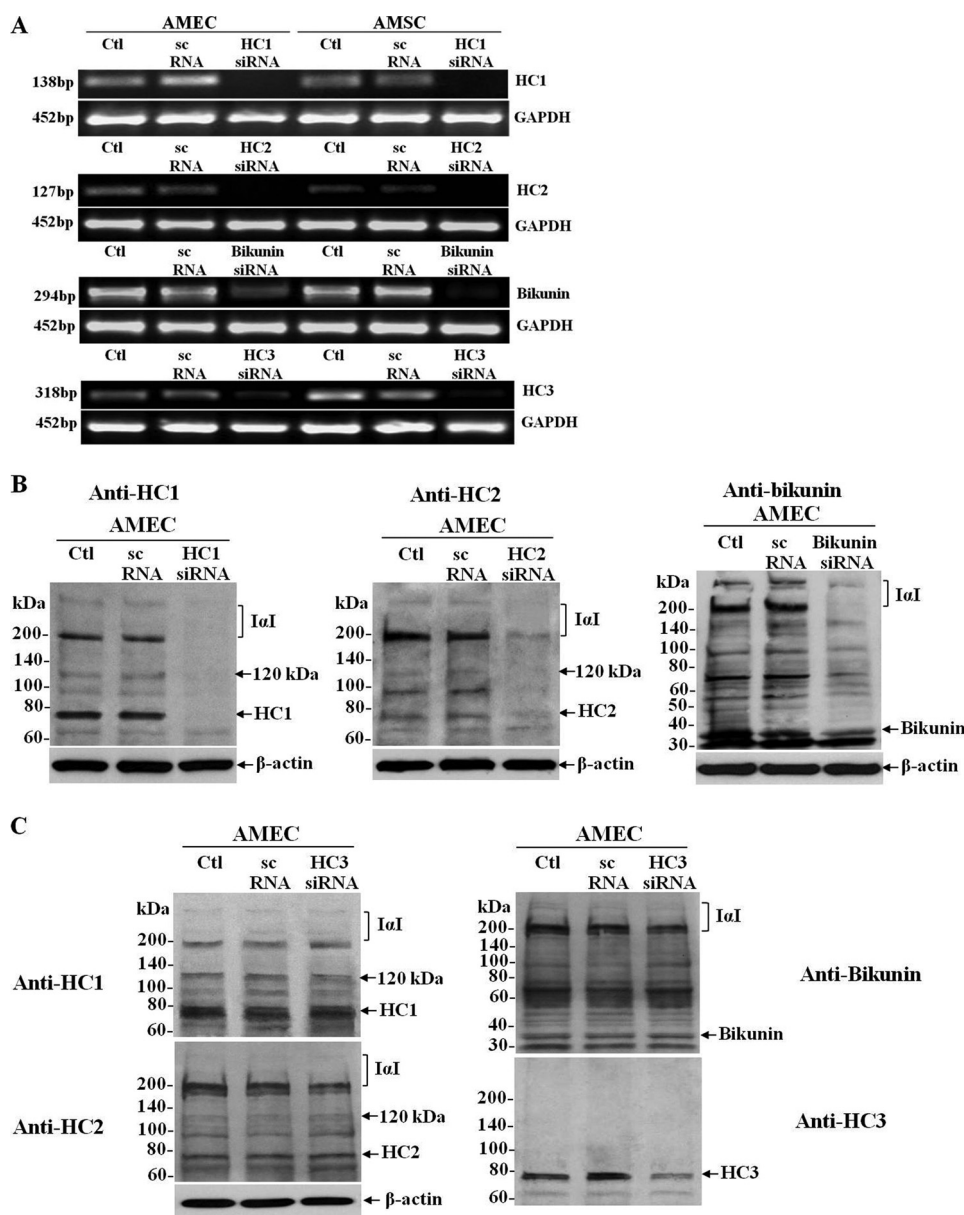


FIGURE 4. Expression of IαI family proteins in serum-free AMECs and AMSCs. Primary AMECs and AMSCs were cultured in serum-free SHEM with or without siRNA to HC1, HC2, bikunin, or HC3. mRNA expression was quantified by RT-PCR using GAPDH as the loading control (A). Total proteins were extracted and subjected to Western blot analysis using antibodies against human HC1, HC2, bikunin (B) and HC3 (C) as indicated. Ctl, control.

*Cellular Production of HC-HA Complex Containing Both HC1 and HC2 whereas AM HC-HA Complex Contains Only HC1*—Previous studies have shown that HC1 and HC2 of IαI and HC3 of PaI can covalently bind to HA *in vivo* and *in vitro* to form HC-HA complex (19, 20, 64). Our prior study showed that a HC-HA complex can be purified from the AM-soluble extract (32). However, it remained unclear whether bikunin or TSG-6 was also present and which HC isotypes were present in AM HC-HA complex. Because AMECs and AMSCs were found here to synthesize their own IαI and TSG-6 proteins (Figs. 4 and 5), we aimed to determine whether they could also produce their own HC-HA complex.

We used two successive ultracentrifugations to isolate the HC-HA complex from the AM extract as reported previously (32) and from both cellular extracts (Fig. 6A). Western blot analysis using the anti-HC1 antibody showed that AM HC-HA

complex presented as a high molecular mass species at the bottom of the loading well and free HC1 (Fig. 6B, lane 3) by comparison with purified IαI treated with NaOH (Fig. 6B, lanes 1 and 2). HAase digestion completely eliminated the high molecular mass species, resulting in a notable increase in the intensity of the HC1 species (Fig. 6B, lane 4), confirming that the high molecular mass species represented a HC-HA complex. The presence of free HC1 in the AM HC-HA complex might be due to the degradation of HA that lead to release of some HC1 during purification and storage of the complex. Interestingly, the anti-HC2 antibody (raised against the N-terminal peptide) (12) did not detect any species in the AM HC-HA complex with or without HAase digestion (Fig. 6B, lanes 9 and 10) but did detect a HC2 species in IαI (Fig. 6B, lane 8). The same result was obtained using an alternative anti-HC2 antibody raised against the C-terminal peptide (12) (Fig. 6B, lanes 13–16). Preliminary

## I $\alpha$ I, TSG-6, and HC-HA Expression by Amniotic Membrane Cells

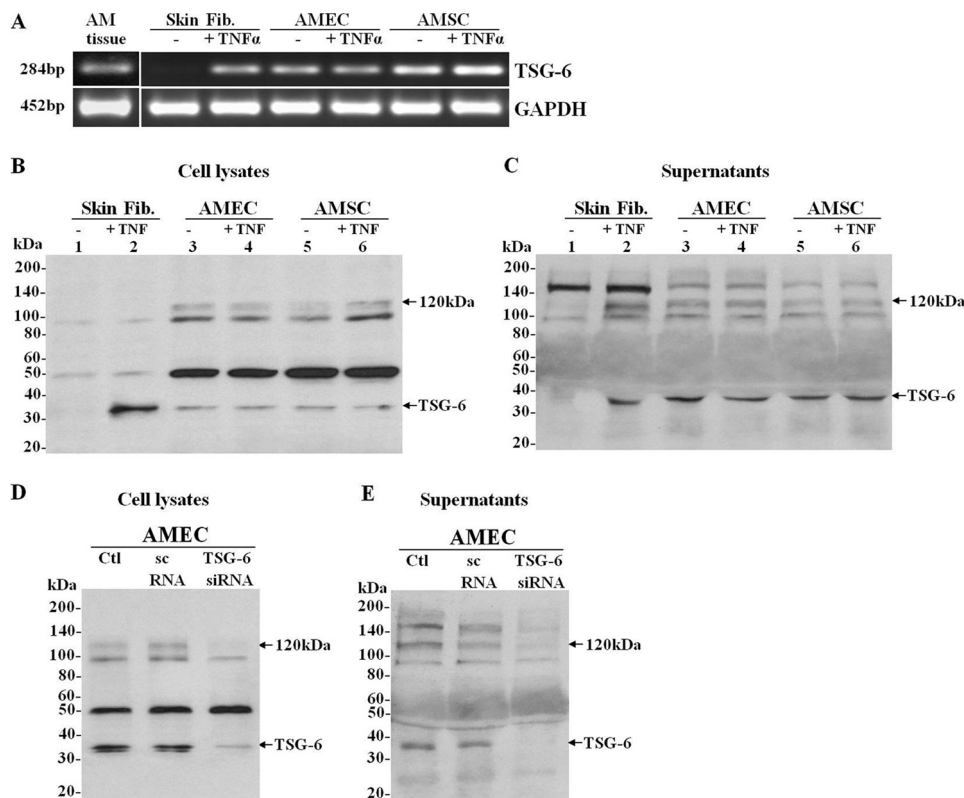


FIGURE 5. **Constitutive expression of TSG-6 mRNA and protein by AMECs and AMSCs.** RNA and protein were extracted from AM tissue, human skin fibroblasts (*Skin Fib.*), and both AMECs and AMSCs cultured in DMEM/F12 plus 10% FBS with or without 20 ng/ml TNF for 4 h (for RT-PCR) or 24 h (for Western blotting). Expression of TSG-6 mRNA (A) and protein in cell lysates (B) and supernatants (C) was compared. TSG-6 siRNA transfection was performed to verify the expression of TSG-6 in AMECs and AMSCs (D and E). *Ctl*, control.

mass spectrometric analysis of the AM HC-HA complex following digestion with HAase and trypsin detected peptides from HC1 but not from HC2 consistent with the absence of HC2 in AM HC-HA complex.<sup>4</sup> Furthermore, by Western blotting we did not find HC3, bikunin, or TSG-6 in the AM HC-HA complex (data not shown); the absence of bikunin and TSG-6 in the HC-HA complex is in agreement with our previous data (32) and with reports on HC-HA obtained from other sources (31, 65).

Overall, the above results indicate that the HC-HA complex from AM only contains HC1. However, Western blot analysis of the aforementioned cell HC-HA revealed the presence of both HC1 and HC2 after HAase digestion of material purified from both AMSCs (Fig. 6B, lanes 5, 6, 11, 12, 17, and 18) and AMECs (data not shown) although the amount increased for HC1 was not as dramatic as HC2. These results indicated that both cells primarily make HC2-HA. We also did not detect HC3, bikunin, and TSG-6 in the cell HC-HA complex (data not shown). The formation of this HC-HA complex was abolished when cells were treated by HC1 siRNA (Fig. 6B, lanes 21 and 22) and TSG-6 siRNA (Fig. 6B, lanes 23 and 24). Available evidence suggests that the covalent coupling of HCs to the CS chain of bikunin to form intact I $\alpha$ I is a prerequisite for the subsequent transfer of HCs to HA (23, 27, 64). We found that HC1-siRNA was specific for HC1 but did not affect HC2 and bikunin mRNA

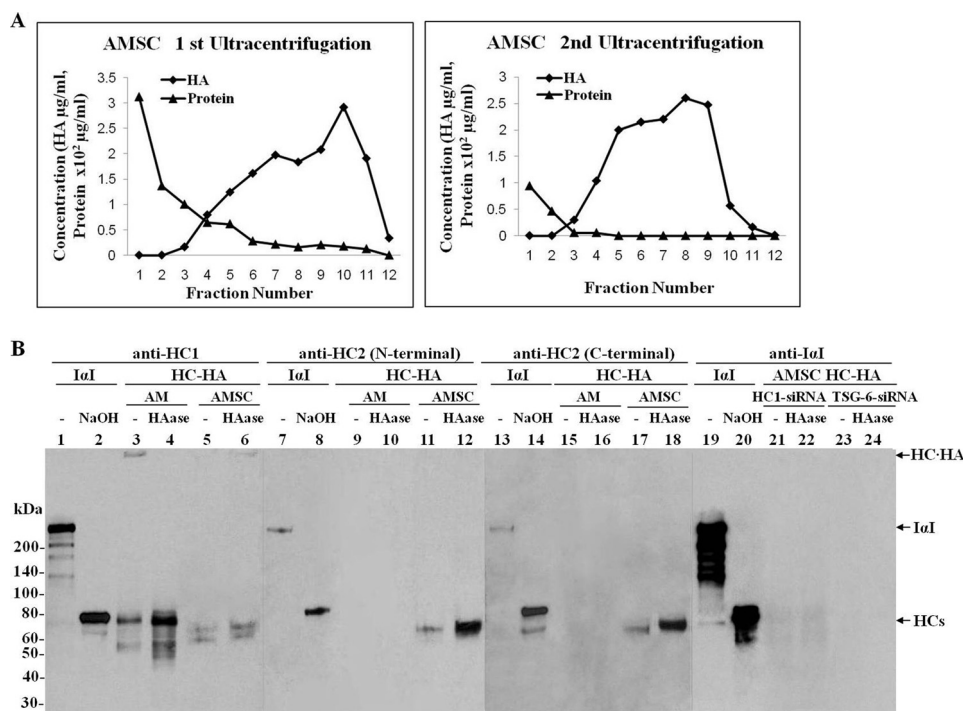
expression and their protein synthesis in AMEC and AMSC (data not shown), but prevented the formation of intact I $\alpha$ I. So, HC1-siRNA treatment prevents any type of HC-HA formation due to the inhibition of I $\alpha$ I biosynthesis caused by HC1 knockdown.

## DISCUSSION

Previous studies have demonstrated that the I $\alpha$ I family proteins are synthesized predominantly by hepatocytes (9, 66) where coupling of HCs and bikunin is accomplished before being secreted into blood circulation (67, 68). Here, for the first time, we have demonstrated that the AM is an extrahepatic tissue that can produce its own I $\alpha$ I family proteins. This assertion was suggested by positive immunostaining to HC1, HC2, HC3, and bikunin in AM cells and matrix (Fig. 1) and by the presence of HC1, HC2, HC3, and bikunin transcripts in AM tissues and the respective proteins in AM extract (Figs. 2 and 5). Furthermore, it was confirmed by the findings that both AMECs and AMSCs expressed transcripts and proteins of HC1, HC2, HC3, and bikunin under a serum-containing or serum-free condition (Figs. 3 and 4). Both 200-kDa and 265-kDa I $\alpha$ I-related species produced by AMECs and AMSCs were cross-reactive with anti-HC1, anti-HC2, and anti-bikunin antibodies but not with the anti-HC3 antibody (Figs. 3 and 4). More importantly, these two I $\alpha$ I-related species were greatly reduced by knockdown with HC1 siRNA, HC2 siRNA, and bikunin siRNA but not HC3 siRNA (Fig. 4). The apparent molecular masses of the two I $\alpha$ I-related species are different from that of

<sup>4</sup> R. D. Unwin, D. C. Briggs, S. Zhang, H. He, S. C. G. Tseng, and A. J. Day, unpublished data.





**FIGURE 6. Production of HC-HA complex in serum-free cultures.** Primary AMECs and AMSCs cultured in serum-free SHEM were treated with or without HC1 siRNA or TSG-6 siRNA. Guanidine-HCl extract of AM cells was subjected to two successive ultracentrifugations with CsCl density gradient and 6 M guanidine HCl (A). The HA-rich and protein-absent fractions were pooled essentially as reported previously for isolation of HC-HA complex from AM extract (32). Cell HC-HA complex and AM HC-HA complex with or without HAase digestion were analyzed by Western blotting using anti-HC1, anti-HC2 (N-terminal and C-terminal) and anti-IαI antibodies (B); purified IαI with or without NaOH treatment was included as a control.

the IαI synthesized by primary hepatocytes (225 kDa) (52) and from the IαI present in AM extract (250 kDa), which is the same size as the IαI control purified from serum. Cultured AMECs and AMSCs produced an IαI with an apparent molecular mass of 200 kDa, which is smaller than that found in AM (Fig. 2). Furthermore, HC1 and HC2 released from cell HC-HA were smaller than those released from serum IαI (and AM HC-HA in the case of HC1) (Fig. 6), as has been found previously for the 70-kDa HC2 from HepG2 cells (52). Such differences are likely explained by differential glycosylation of IαI from different cell sources (69).

Previous studies have shown that TSG-6 mRNA and protein are not constitutively expressed in many adult cells but is induced by pro-inflammatory cytokines such as IL-1 and TNF (for review, see Ref. 26). Indeed, cultured adult human skin fibroblasts did not express TSG-6 until TNF was added (Fig. 5). In contrast, for the first time, we noted that TSG-6 mRNA was constitutively expressed in fresh AM tissue (Fig. 5), and TSG-6 protein was detected in AM extract (Fig. 2). Moreover, TSG-6 mRNA and protein were constitutively expressed by AMECs and AMSCs without being significantly up-regulated by TNF (Fig. 5). It has also been found that TSG-6 is constitutively expressed by human neutrophils (and then stored in specific granules) (59) and is present in murine bone marrow (this probably corresponds to the presence of polymorphonuclear leukocytes) (70), in human intervertebral disc (71), in human skin (72), and in human periprosthetic breast capsule tissue (73); TSG-6 may play a protective role in some or all of these settings. Because the AM has been shown to be an excellent source of mesenchymal stem cells (for review, see Refs. 74, 75) and

because expression of TSG-6 has been reported to be a hallmark for delivering the therapeutic effect of engrafted mesenchymal stem cells (e.g. in a model of myocardial infarction) (76), the constitutive expression of TSG-6 in AM may also contribute to an anti-inflammatory and protective role in the ocular surface.

TSG-6, however, may have other more crucial role in AM. One well known action of TSG-6 is to catalyze the trans-esterification reactions that form the HC-HA complex (11, 21–23); that is, HCs of IαI are transferred from CS to HA with the release of bikunin. For the HC-HA complex found in the cumulus matrix of preovulatory follicles (77–79) and the synovial fluid of rheumatoid arthritis patients (20, 29), the source of HCs is believed to be from serum IαI. The constitutive presence of both IαI and TSG-6 in AM extract and production by both AMECs and AMSCs in serum-free cultures (Figs. 4 and 5) suggest that the HC-HA complex can endogenously be produced by these two AM cells without serum participation. Indeed, the HC-HA complex was purified by two successive ultracentrifugations from the guanidine-HCl-extracted lysates in serum-free AMEC and AMSC cultures (Fig. 6). Furthermore, the production of such an HC-HA complex was abolished when cells were treated with siRNA to TSG-6 or HC1 (Fig. 6). In cumulus matrix and pathological synovial fluid, all three HC isoforms (HC1, HC2, and HC3) have been found to form covalent complexes with HA (18, 20). Our study showed that both HC1 and HC2 were incorporated in cell HC-HA complex, whereas only HC1 was covalently bound to HA in the AM HC-HA complex (Fig. 6). The absence of HC2 was verified by both anti-C- and anti-N-terminal antibodies and was consist-

ent with our initial mass spectrometric analysis. Future studies are needed to delineate how the *in vivo* biosynthetic environment might favor the formation of the HC-HA complex with specific compositions (e.g. just HC1) to yield unique physiological actions.

The HC-HA complex purified from AM exerts potent anti-inflammatory, anti-scarring, and anti-angiogenic actions (32, 33), explaining why transplantation of cryopreserved AM is effective for treating a number of ocular surface diseases. Herein, our study provides strong evidence that the AM can produce endogenous I $\alpha$ I and TSG-6 proteins that can support the formation of its own HC-HA complex. Given that AM is avascular, locally produced HC-HA complex may play a crucial protective role during fetal development.

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## $I\alpha I$ , TSG-6, and HC-HA Expression by Amniotic Membrane Cells

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