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Basement Membrane Heparan Sulfate Proteoglycan (Perlecan) Synthesized by ACC3, Adenoid Cystic Carcinoma Cells of Human Salivary Gland Origin[1]

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The biosynthesis of basement membrane heparan sulfate proteoglycan (HSPG), known as perlecan, in ACC3 cells established from a adenoid cystic carcinoma of the human salivary gland was studied using metabolic labeling and immunoprecipitation with discriminative antibodies specific for HSPG core protein. Treatment of immunoprecipitated HSPG with HNO_2, heparitinase, and chondroitinase ABC revealed that ACC3 cells synthesized HSPG molecules composed of 470-kDa core protein and heparan sulfate but not of chondroitin sulfate. The core protein was shown to contain complex type *N*-linked oligosaccharides by digestion with *N*-glycanase and endoglycosidase H. Pulse-chase experiments showed that the mature form of HSPG was formed in the cells in 30 min and released into the medium thereafter. Degradation of HSPG was also found in the chase period of 3 h. In time course experiments, HSPG was found to be synthesized maximally at day 4 after plating, deposited in the cell layer maximally at day 6, and secreted maximally at day 8. This was also confirmed by immunofluorescence, Northern blotting, and *in-situ* hybridization. The results indicate that ACC3 cells synthesize, secrete and degrade basement membrane type HSPG, which is analogous to those produced by other cell types, and that the biosynthesis and secretion of HSPG in ACC3 cells are strictly regulated by the cell growth, that may be reflected in the characteristic histology of adenoid cystic carcinomas.

Key words: adenoid cystic carcinoma, basement membrane, extracellular matrix, heparan sulfate proteoglycan, perlecan.

Basement membranes are defined electron microscopically as characteristic sheet-like structures which separate the cells residing on them from underlying interstitial connective tissue. Basement membranes are composed of at least four major macromolecules: type IV collagen, laminin, entactin, and heparan sulfate proteoglycan (HSPG) (*1*). It has been proposed that basement membranes serve as a barrier for tumor invasion, and destruction of basement membranes by tumor cells is the first step of tumor invasion and metastasis (*2*). Recent studies have revealed that the constituent molecules of basement membranes are expressed not only in the basement membrane but also in the stromata of neoplastic or inflammatory tissues (*3-6*). These data may imply that basement membrane molecules function in the proliferation of tumor cells or stromal cells as well as in the tissue remodeling during tumor invasion or granulation formation.

Basement membrane type HSPGs which have core proteins ranging from 350 to 500 kDa in size have been isolated from several sources and characterized as follows: mouse EHS tumors (*7*), endothelial cells (*8*), epithelial cells (*9*), human colon carcinoma cells (*10*), and fibroblasts (*11*). Recently, the primary structure of the large human basement membrane type HSPG core protein was determined from human and mouse cDNA clones, and several biological functions of HSPG core proteins were suggested from their multidomain structures with possible attachment sites for heparan sulfate (HS) chains and oligosaccharides (*12, 13*). However, their functional roles and metabolic processes, especially those of their core proteins, are poorly understood.

Adenoid cystic carcinomas arising in human salivary glands are histopathologically characterized by cribriform structures formed by pseudocystic spaces. The pseudocystic spaces are abundant in basement membrane molecules, especially HSPG (*3*), and these molecules has been shown to be synthesized by parenchymal cells of adenoid cystic carcinomas (*14-18*). We have shown that basement membrane molecules including HSPG were synthesized and secreted by ACC2 and ACC3 cells, which were established independently from human adenoid cystic carcinomas (*19*). As for HSPG, we have also demonstrated its intracytoplasmic pathway in ACC3 cells by immunoelec-

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Abbreviations: DIG, digoxigenin; MEM, minimum essential medium; SSC, saline sodium citrate.

tron microscopy (*20*). Based on these data, we have suggested that the characteristic histology of adenoid cystic carcinomas resulted from the overproduction of basement membrane molecules by the tumor cells, and that these cell systems should be valuable sources of human basement membrane molecules because other cell lines with basement membrane molecule production potential have not been established. However, the molecules synthesized by ACC2 or ACC3 cells have not yet been characterized biochemically.

In this study, we carried out biosynthetic labeling experiments and detection of mRNA to demonstrate the biosynthesis, secretion and degradation of basement membrane type HSPG in ACC3 cells. The data reveal that the molecule has a 450 kDa core and a total of 20 kDa complex type *N*-linked oligosaccharides when it is secreted into the medium.

MATERIALS AND METHODS

Cells—The cell system, ACC3, was established from an adenoid cystic carcinoma arising in a human parotid gland as previously described (*19*). The cells were cultured in RPMI-1640 medium (Nissui, Tokyo) containing 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, CA), 1% glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin, and incubated at 37°C under a humidified 5% CO_2/95% air atmosphere.

Antibody—The antibody to the HSPG core protein was raised in rabbits by immunization with that purified from the culture medium of bovine aortic endothelial cells as previously described (*8*).

Immunofluorescence Staining—Cells at a concentration of 3×10^4 in 2-ml medium were plated on a 35 mm plastic dish on which a piece of cover glass had been placed. The dishes were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min on ice every 48 h for 8 days after plating, and then permeabilized by adding 0.2% Triton X-100 to the fixative for 20 min on ice. After overnight treatment with 5% skim milk in PBS (pH 7.4) containing 0.05% Triton X-100 to prevent non-specific protein binding, the dishes were stained by means of an indirect immunofluorescence technique. They were incubated with the rabbit antibodies against bovine HSPG core protein and rhodamine-conjugated goat anti-rabbit IgG (1:50; Miles Scientific, Naperville, IL) for 1 h each at room temperature. After immunostaining, the cover glasses were removed from the dishes and mounted on slide glasses with 90% glycerol in PBS. As a control, preimmune rabbit IgG was used instead of the specific primary antibodies. The dishes were examined under a phase contrast microscope equipped with epifluorescence optics and an automatic camera system (Olympus BH-2, PM-10AD, Tokyo). Representative slides were photographed on Fujichrome 400 film.

Cell Labeling and Pulse-Chase Experiments—For labeling experiments, cells cultured for 6 days were preincubated with methionine-free or SO_4-free minimum essential medium (MEM) for 1 h and then incubated in fresh MEM containing 50-100 μCi of [^{35}S]methionine or [^{35}S]sulfate (Du Pont-New England Nuclear, Boston, MA) for 3 h. For the pulse-chase experiments, cells were preincubated for 1 h at 37°C in MEM lacking methionine and calf serum. The cells were then pulse-labeled with 250 μCi/ml of [^{35}S]-methionine for 30 min and chased in the complete MEM for 30 min, 1 and 3 h. After removing the medium, the cells were lysed in 0.5 ml of a lysis buffer [10 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl]. Then a protease inhibitor mixture containing 1 mg/ml each of antipain, aprotinin, chymostatin, elastatinal, leupeptin, pepstatin A, and phosphoramidone was added to the lysates and media at a final concentration of 10 μg/ml each. Both the cell lysates and media were centrifuged at $15,000\times g$ for 10 min, and the resultant supernatants were subjected to immunoprecipitation.

Immunoprecipitation—The cell lysates and media were shaken with 50 μl of fixed *Staphylococcus aureus* cells (Pansorbin®; Calbiochem-Novabiochem Intl, La Jolla, CA) for 30 min and then centrifuged. Precleared lysates and media were adjusted to a final concentration of 0.5% with skim milk and then incubated with 5 μl of the antibodies against the bovine HSPG core protein overnight on a shaker. Immune-complexes were isolated by shaking for 1 h with 40 μl of a 50% suspension of protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) in PBS. The protein A-Sepharose beads were washed three times with the lysis buffer, three times with NHSTE buffer [10 mM Tris-HCl (pH 7.5) containing 0.5% Nonidet P-40, 500 mM NaCl, and 2 mM EDTA], twice with NSTE (containing 150 mM NaCl instead of 500 mM NaCl of NHSTE), and once with 10 mM Tris-HCl (pH 7.5) containing 0.1% SDS and 1 mM EDTA. All procedures were performed at 4°C. The immunoisolated materials were dissolved in Laemmli's sample buffer (*21*), boiled for 5 min, centrifuged at $10,000\times g$ for 5 min to remove beads, and then analyzed by SDS-polyacrylamide gel electrophoresis.

HNO_2 Treatment and Enzyme Digestion—Nitrous acid treatment at a low temperature was performed according to Shively and Conrad (*22*). Immunoprecipitated materials were treated with HNO_2 for 10 min at 0°C and then neutralized with a 3 M Tris solution. All enzymatic digestions were performed at 37°C in the presence of the protease inhibitor mixture described above and 0.1 mM *p*-amidinophenyl methanesulfonyl fluoride. Chondroitinase ABC (Seikagaku Kogyo, Tokyo) digestion was carried out for 5 h in 100 mM Tris-HCl (pH 7.4) containing 0.1 U/ml enzyme and 2 mM EDTA. Heparitinase (Seikagaku Kogyo) digestion was performed overnight in 10 mM Tris-HCl (pH 7.4) containing 0.1 U/ml enzyme, 130 mM NaCl, and 4 mM $CaCl_2$. Digestions with peptide-*N*-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) and endo-β-*N*-acetylglucosaminidase H (Endo-H) (New England Biolabs) were performed for 3 h under the following conditions: 0.15 U/ml of PNGase F in 50 mM Tris-HCl (pH 7.5) or 0.15 U/ml of Endo-H in 50 mM acetate buffer (pH 5.5). HNO_2-treated and enzyme-digested materials were dissolved in Laemmli's sample buffer and processed as described above.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out in slab gels with 5% acrylamide according to Laemmli (*21*), followed by fluorography (*23*) using a Hyperfilm-MP™ X-ray film (Amersham International plc, Buckinghamshire, England). Apparent molecular weights were determined by co-electrophoresis of marker proteins.

Preparation of RNA Probes—Perlecan RNA probes were prepared with a digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) using SP6/T7 RNA polymerase (Promega Corporation, Madison, WI). Template cDNA (1.5 kbp, corresponding to domain III of human perlecan) was generously provided by Dr. Karl Tryggvason (*12*). The cDNA was digested with *Eco*RI and *Sma*I, and the resultant 700 bp fragment was ligated into the pSPT18 vector (Boehringer Mannheim). The vector plasmid was linearized with *Sma*I and then transcribed with T7 RNA polymerase as an antisense probe, or was linearized with *Eco*RI and then transcribed with SP6 RNA polymerase as a sense probe.

Total RNA Isolation and Northern Blot Analysis—Total RNA was prepared from cultures of ACC3 cells every 48 h for 8 days with ISOGEN® total RNA isolation reagent (Nippongene, Tokyo) according to the manufacturer's instructions. The total RNA was electrophoresed on a 0.8% agarose gel containing formaldehyde, transferred to a nitrocellulose filter and then hybridized with 100 ng/ml of a DIG-labeled RNA probe prepared as described above at 65°C for 16 h. The hybridized probes were detected with DIG detection kits (Boehringer Mannheim) using alkaline phosphatase-conjugated sheep anti-DIG antibodies.

In-Situ Hybridization—Cells cultured for 6 days on cover glasses were fixed in 4% paraformaldehyde for 15 min. The fixed cells were washed with PBS, acetylated with 5% acetic anhydride in ethanol, and then air-dried. After treatment with a series of ethanol solutions (100 to 30%), the cells were treated with 1 μg/ml of proteinase K for 20 min at 37°C. They were then treated with 0.2% glycine in PBS, dehydrated with a series of ethanol (70 to 100%), and air-dried. Hybridization was performed at 45°C for 16 h in a moist chamber. The hybridization solution contained 10 mM phosphate buffer (pH 7.4), 10% dextran sulfate, 1× Denhardt's solution, 100 μg/ml of salmon sperm DNA, 125 μg/ml of yeast tRNA, 3×SSC, 50% formamide, and 500 ng/ml of probe. After hybridization, the cover glasses were rinsed in 2×SSC and then the hybridized probes were detected with DIG detection kits (Boehringer Mannheim).

Quantitative Analysis—The fluorograms or blotted sheets were scanned with a Sharp flatbed scanner JX-320M (Sharp, Tokyo), and then the images were saved on a personal computer. The scanned images were analyzed and the relative amount of HSPG was determined with NIH Image, an image processing and analysis program.

RESULTS

Immunofluorescence Staining for HSPG—From days 2 to 4 after seeding, diffuse or fine granular immunofluorescence signals for HSPG were observed in the cytoplasm (Fig. 1, a and b), indicating that the main immunolocalization of HSPG was in the rough endoplasmic reticulum (ER), and the level of production of HSPG in ACC3 cells was still low. On day 6, when the cells had spread widely and reached their subconfluent proliferation level, the signals were largely concentrated in the perinuclear area of most of the cells (Fig. 1c). We interpreted this as that HSPG molecules had been abundantly synthesized in the rER, modified in the Golgi apparatus, and sorted into secretory vesicles, and were ready for secretion. On day 8, when the cells had reached confluency, signals appeared in the intercellular space, whereas the intracellular staining was diminished (Fig. 1d). The intercellular signals were regarded as extracellular deposition of HSPG secreted by the cells.

Biosynthesis of HSPG—To detect HSPG and its core protein, ACC3 cells were metabolically labeled with [^{35}S]-methionine or [^{35}S]sulfate, and then the media were immunoprecipitated with antibodies against basement membrane HSPG core protein. HSPG appeared as smear bands largely at the top of the resolving gel (Fig. 2, lanes 1 and 3). This broad pattern of HSPG molecules was presumably due to the heterogeneity of heparan sulfate chains, since only a single band corresponding to a molecular mass of 470 kDa was detected after nitrous acid treatment (Fig.

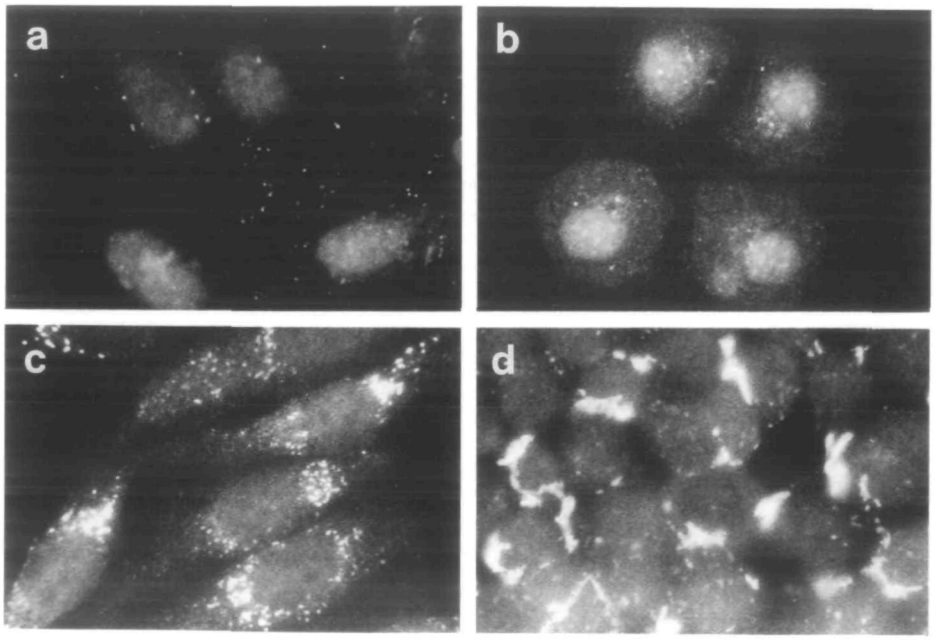

Fig. 1. **Immunofluorescence for HSPG in ACC3 cells.** a, day 2. b, day 4. c, day 6. d, day 8. Signals in the endoplasmic reticulum (a, b) and the Golgi apparatus (c) change into the extracellular space (d) with growth of the cells.

2, lane 2), which eliminated [^{35}S]sulfate-labeled glycosaminoglycan chains from the core protein of HSPG (Fig. 2, lane 4). Similar to nitrous acid treatment, digestion with chondroitinase (Fig. 3, lanes 2 and 5) or heparitinase (Fig. 3, lanes 3 and 6) demonstrated that the glycosaminoglycan chains of HSPG synthesized by ACC3 are heparan sulfate.

Time Course of HSPG Biosynthesis—As shown in Fig. 1, the immunofluorescence pattern of HSPG in ACC3 cells changed remarkably with the growth of ACC3 cells throughout the experimental period. To study such biosynthetic dynamics biochemically, ACC3 cells were labeled with [^{35}S]methionine every 48 h for 8 days after plating. The labeled HSPGs were immunoprecipitated from the cell lysates and media with the anti-HSPG core protein antibodies, and then were analyzed as described under "MATERIALS AND METHODS." On day 2, [^{35}S]methionine-labeled HSPG was faintly detected in the gel interface in the cell layer (Fig. 4a, lane 1), but not in the medium (Fig. 4b, lane 1). On day 4, HSPG became more distinct in the cell layer (Fig. 4a, lane 2), being only faint in the medium (Fig. 4b, lane 2). Compatible with the morphological observation shown in Fig. 1, HSPG molecules were produced maximally in the cells and secreted out of them on day 6 (Fig. 4, a and b, lane 3). On day 8, still producing HSPG within them, ACC3 cells secreted it more into the medium, corresponding to the increased intercellular deposition and decreased

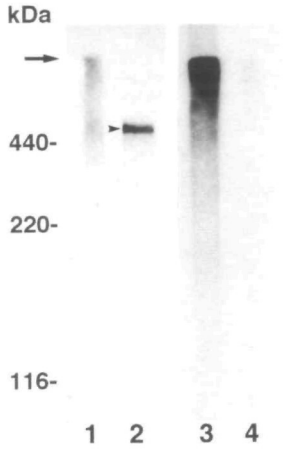

Fig. 2. **Detection of HSPG and its core protein in 6 days culture medium on immunoprecipitation and HNO_2-treatment.** ACC3 cells were labeled with [^{35}S]methionine (lanes 1 and 2) or [^{35}S]-sulfate (lanes 3 and 4) for 3 h, and then harvested, lysed, and immunoprecipitated, for HSPG. The immunoprecipitates before (lanes 1 and 3) and after HNO_2-treatment (lanes 2 and 4) were analyzed by SDS-polyacrylamide gel electrophoresis/fluorography. The arrow indicates the interface of the stacking gel and the arrowhead indicates the HSPG core protein. Molecular markers, laminin A chain (440 kDa), laminin B chain (220 kDa), and β-galactosidase (116 kDa), were electrophoresed on the same gel, and the gel was stained with Coomassie brilliant blue before fluorography.

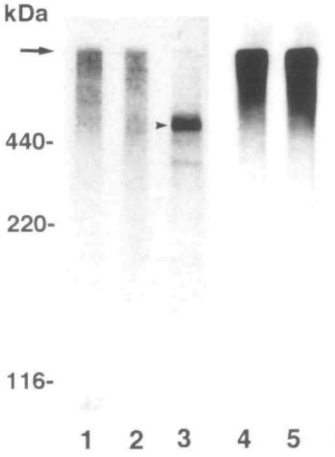

Fig. 3. **Digestion of immunoprecipitated HSPG by chondroitinase and heparitinase.** ACC3 cells were labeled with [^{35}S]methionine (lanes 1-3) or [^{35}S]sulfate (lanes 4-6), and then immunoprecipitated. The immunoprecipitates were incubated in the absence (lanes 1 and 4) or presence of chondroitinase ABC (lanes 2 and 5) or heparitinase (lanes 3 and 6), and analyzed as shown in Fig. 2. The arrow indicates the interface of the stacking gel and the arrowhead indicates the HSPG core protein.

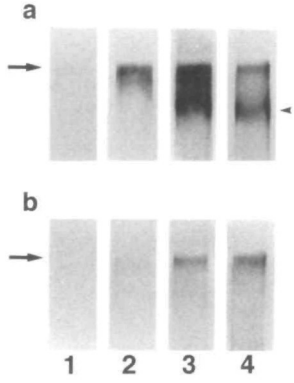

Fig. 4. **Time course of biosynthesis of HSPG.** ACC3 cells were labeled for 5 h with [^{35}S]methionine at every 48 h after plating for 8 days, and then the medium (panel b) and cell lysates (panel a) were immunoprecipitated and analyzed as shown in Fig. 2. Lane 1, day 2; lane 2, day 4; lane 3, day 6; lane 4, day 8. Arrows indicate the interface of the stacking gel. The arrowhead indicates the 480-kDa core protein.

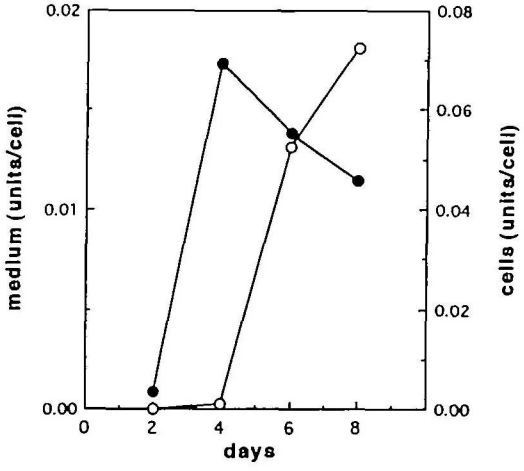

Fig. 5. **Quantitative analysis of the data obtained in time course experiment shown in Fig. 4.** The relative amounts of HSPG in the cell layer (closed circles) and the medium (open circles) were determined from the scanning data. Units indicate the relative amounts per cell calculated with the image analysis program, NIH Image.

Golgi pattern shown by the immunofluorescence (Fig. 1d). It is noteworthy that a band corresponding to the smaller molecular mass (=480 kDa) of the core protein of HSPG was observed for the cells after day 6 (Fig. 4a, lanes 3 and 4).

Quantitative analysis of the production of HSPG as a function of time showed that the biosynthesis of HSPG started within 2 days after inoculation and became maximum on day 4, and then decreased gradually from day 6 to 8 (Fig. 5). In contrast, the increase in the medium HSPG started two days after that in the cell layer one, and its radioactivity was observed until day 8. The results indicate that ACC3 cells actively synthesized and secreted HSPG on days 4 to 6, whereas the secretion and shedding of HSPG into the medium occurred after some delay on days 6 to 8. These two experiments showed that the time course of HSPG biosynthesis seemed to correspond with the cell growth.

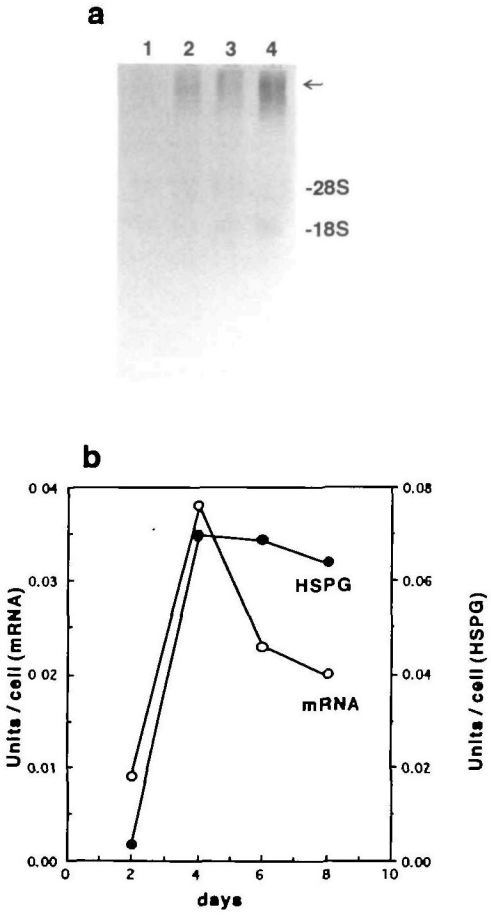

Fig. 6. **Northern blot analysis of HSPG.** Panel a, total RNA was prepared every 48 h for 8 days, electrophoresed, blotted, and hybridized with an antisense RNA probe as described under "MATERIALS AND METHODS." Lane 1, day 2; lane 2, day 4; lane 3, day 6; lane 4, day 8. The arrow indicates the 14-kbp mRNA for HSPG, and the positions of 28S and 18S rRNA are also indicated. Panel b, quantitative analysis of the data in panel a. The relative amounts of HSPG mRNA per cell (open circles) and of HSPG core protein (closed circles, determined from the data in Fig. 4) were determined from the scanning data. Units indicate the relative amounts per cell calculated with the image analysis program, NIH Image.

To confirm the results of the time course experiment, expression of mRNA for HSPG was determined by Northern blotting. An about 14-kbp mRNA for HSPG was detected in the experimental time course from day 2 to 8 (Fig. 6a), and the signals were quantitatively analyzed as the relative amount per cell (Fig. 6b). The expression of HSPG mRNA per cell was maximum on day 4, which was

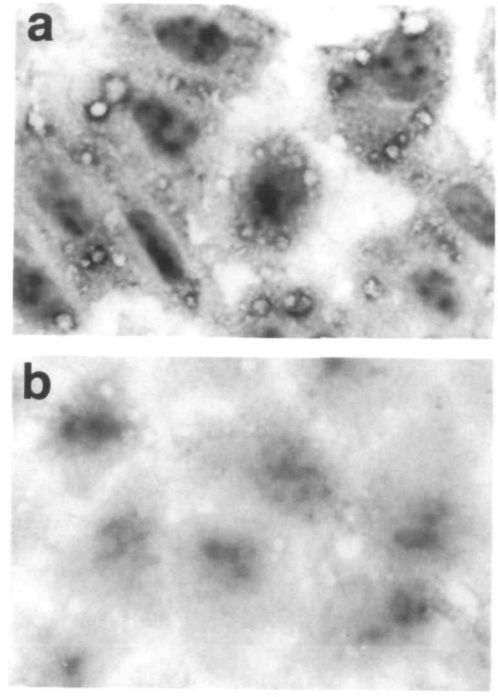

Fig. 7. ***In-situ* hybridization for HSPG mRNA.** ACC3 cells fixed on day 6 were hybridized with antisense (a) and sense (b) RNA probes as described under "MATERIALS AND METHODS." Positive signals are diffusely demonstrated in the cytoplasm, especially around vacuolar structures.

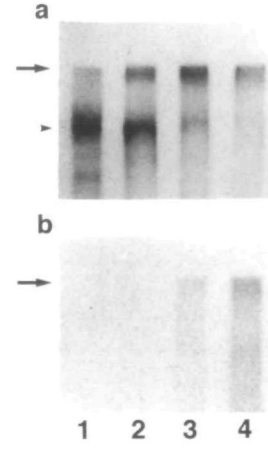

Fig. 8. **Process of maturation of HSPG.** ACC3 cells were labeled for 30 min with [^{35}S]methionine (lane 1), and then chased for 30 min (lane 2), 60 min (lane 3), or 180 min (lane 4) in the medium containing unlabeled methionine. At the indicated times, immunoprecipitates of HSPG were prepared and analyzed as shown in Fig. 2. Panel a, cell layer; panel b, medium. Arrows indicate the interface of the stacking gel and the arrowhead indicates the HSPG core protein.

consistent with the result as to synthesis of the HSPG core described above. Furthermore, the expression of mRNA for HSPG within the ACC3 cells on day 6 was clearly confirmed by *in-situ* hybridization (Fig. 7). Hybridization signals for HSPG mRNA were diffusely observed in the cytoplasm, especially around vacuolar structures.

Kinetics of Glycosylation of the HSPG Core—We further examined the process of biosynthesis of HSPG molecules in ACC3 cells by means of a pulse/chase experiment. When ACC3 cells on day 6 were pulse-labeled with [^{35}S]methionine for 30 min, a major band corresponding to a molecular mass of approximately 470 kDa, corresponding to the core protein of HSPG, and several additional bands of smaller molecular weights were detected for the cell layer (Fig. 8a, lane 1). The additional bands were regarded as representing prematurely degraded forms of the core protein, because the core protein was shown to decrease rapidly in this pulse-chase experiment described as follows. At 30 min after the start of the chase, the radioactivity for the core protein of HSPG had slightly decreased, but the mature form of HSPG appeared intensively at the gel interface (Fig. 8a, lane 2). At 1 h of chase, the radioactivity for the core protein had decreased markedly, whereas the mature form increased instead (Fig. 8a, lane 3). After 3 h, the core protein disappeared completely and only a small amount of the intact HSPG was left in the cell layer (Fig. 8a, lane 4). In the medium, there was no radioactivity for HSPG until 1 h of chase (Fig. 8b, lanes 1 and 2), when a small amount of the mature HSPG first appeared at the gel interface (Fig. 8b, lane 3). The mature form in the medium had increased significantly at 3 h (Fig. 8b, lane 4). As observed on densitometric analysis, the 470-kDa core protein started to be converted to the mature form of HSPG within 30 min after the start of pulse-labeling and 70% of the conversion occurred within 1 h of chase (Fig. 9a). The core protein had a half-life of about 50 min. Quite interestingly, there was a 70% loss of the total amount of [^{35}S]-methionine-labeled HSPG within 3 h. This suggests that a significant amount of newly synthesized HSPG molecules was degraded within the cells (Fig. 9b).

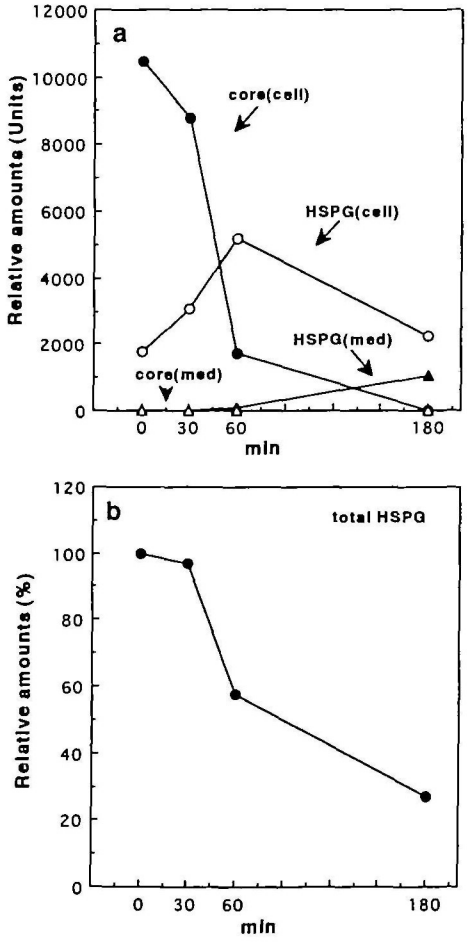

Fig. 9. **Quantitative analysis of the data obtained in the pulse-chase experiment shown in Fig. 6.** Panel a, relative amounts of HSPG (closed circles) and core protein (closed triangles) in the cell layer, and those of HSPG (open circles) and the core protein (open triangles) in the medium were determined from the scanning data as shown in Fig. 5. Panel b, total amount of HSPG calculated from the data in panel a. Units indicate the relative amounts per cell.

To determine if the core protein of HSPG was glycosylated with *N*-linked oligosaccharides, the [^{35}S]methionine-labeled core protein was digested with Endo-H or PNGase F (Fig. 10). As shown above (Fig. 8a), the core protein without the addition of heparan sulfate was obtained by immunoprecipitation from lysates of cells labeled for 30 min with [^{35}S]methionine (Fig. 10a, lane 1). After digestion with Endo-H, the core protein migrated faster than without Endo-H treatment on the gel (Fig. 10a, lane 2), indicating that the core protein contained Endo-H sensitive, high-mannose type *N*-linked oligosaccharides. When the mature form of HSPG secreted into the medium (Fig. 10b, lane 1) was digested with heparitinase to remove heparan sulfate chains (Fig. 10b, lane 3) and then sequentially treated with either Endo-H or PNGase F, the secreted core protein was found to be resistant to Endo-H (Fig. 10b, lane 4) but sensitive to PNGase F (Fig. 10b, lane 5). The results indicated that the core protein undergoes processing of *N*-linked oligosaccharides from the high-mannose type to the complex type during passage through the intracellular secretory pathway. Since digestion with chondroitinase ABC did not affect the [^{35}S]methionine-labeled core protein of HSPG (Fig. 10b, lane 2), glycosaminoglycan chains attached to the core protein were shown to comprise heparan sulfate but not chondroitin sulfate, as

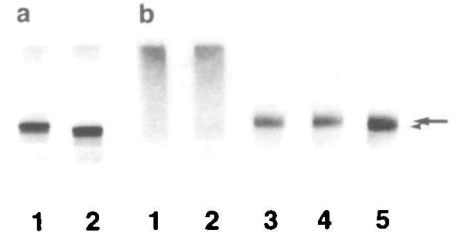

Fig. 10. **Demonstration of *N*-linked oligosaccharide chains in HSPG.** Panel a, the core protein was immunoprecipitated from ACC3 cells labeled with [^{35}S]methionine for 30 min (lane 1), and then treated with Endo-H (lane 2). Panel b, HSPG immunoprecipitated from the culture medium of ACC3 cells labeled with [^{35}S]methionine for 3 h (lane 1) was treated sequentially with chondroitinase ABC (lane 2) and heparitinase (lane 3). The resulting core protein was treated with Endo-H (lane 4) or PNGase F (lane 5). The arrow indicates the HSPG core protein of 470 kDa and the arrowhead indicates the core protein of 450 kDa.

also revealed in the experiment using [^{35}S]sulfate labeling (Fig. 3).

DISCUSSION

Previous immunohistochemical and immunocytochemical studies have shown that ACC2 and ACC3 cells, which were established from human adenoid cystic carcinomas independently, produce HSPG and other basement membrane molecules (*19, 20, 24*). Characterization of HSPG and other molecules should be the next and an essential step for understanding the characteristic histological architecture, and clinical and biological behavior of adenoid cystic carcinomas. The present study has examined biochemically the synthesis of HSPG in the ACC3 cell system.

The biochemical data obtained on metabolic labeling with [^{35}S]sulfate and [^{35}S]methionine indicated that ACC3 cells synthesize high molecular weight HSPG, which is similar in size to those of other cells (*7, 9-11*). Treatment with HNO_2 and heparitinase revealed that the molecular mass of the HSPG core was 470 kDa, which is consistent with M_r 466,876 (*12*) and 466,564 (*13*) calculated from the amino acid sequences of the human fibrosarcoma and human colon HSPGs, respectively. It is also similar to that of the 400-kDa core protein of HSPG synthesized by human colon carcinoma cells (*25*), mouse mammary gland cells (*26*), and mouse EHS tumor (*27*). The molecular mass of the core protein is also similar to that of that synthesized by bovine aortic endothelial cells, which we previously determined to be 500 kDa (*8*). We demonstrated in this study that this HSPG molecule from ACC3 cells contains only heparan sulfate chains, *i.e.* no chondroitin sulfates, although we did not characterize its heparan sulfate chains further. These results suggest that the core proteins of basement membrane type HSPGs are homologous regardless of the cell type or species. As the source of human basement membrane type HSPG, the molecule from ACC3 cells could be the most valuable, as far as we could determine by searching the literature. Adenoid cystic carcinomas have also been shown to be a useful source for human laminin and type IV collagen when they have been xenografted into nude mice (*28*).

Our immunohistochemical study using confocal microscopy (*19*) has already suggested that the immunolocalization pattern of HSPG in ACC3 cells changed concomitantly with cell growth. This was confirmed biochemically by the present time course experiments. The time course study of HSPG production showed that the synthesis of HSPG had increased rapidly on day 4 after plating and the secretion and/or shedding of HSPG became significant on days 6 to 8 after plating. These observations were also compatible with our previous data obtaind on immunoelectron microscopy (*20*). The immunocytochemical study revealed that the immunofluorescence of HSPG concentrated in the Golgi area on day 6 did not always imply its actual localization only in the Golgi apparatus. HSPG molecules were at the same time immunolocalized in the rER with dilated cisternae, secretory vesicles, multivesicular bodies and lysosomes. This indicated that some of the HSPG molecules transported to the Golgi apparatus might be degraded before they were secreted or shed into the medium. This was confirmed biochemically in the present study, as shown in Figs. 4 and 8. The extracellular immunofluorescence of HSPG increased with time during the culture of ACC3 cells, while its minimal deposition was observed immunocytochemically from day 1 of culture. Taken together, these findings indicate that the synthesis and secretion of HSPG in ACC3 cells are regulated by their cell growth. Furthermore, the results of Northern blot analysis strongly support this idea. The maximal increase in the relative amount of mRNA for HSPG per cell on day 4 and the decrease thereafter were almost parallel to the mode of synthesis of the HSPG core. The expression of mRNA for HSPG was morphologically confirmed by *in-situ* hybridization. Interestingly, the HSPG mRNA signals concentrated around vacuolar structures may indicate that the mRNA was localized in the rER around dilated Golgi cisternae. Such dilated vacuolar structures have been shown to be characteristic of ACC3 cells by immunoelectron microscopy (*20*).

Pulse/chase experiments showed that the core protein of HSPG started to be converted to the mature form within 30 min after labeling. The conversion was mostly completed within 90 min. The 50 min half-life of the core protein was shorter than that of human colon carcinoma cells (*25*). The findings that of the shedding of HSPG into the culture medium became apparent at 3 h but that its total amount decreased in 3 h suggest the existence of intracellular and/or extracellular degradation pathways for the core protein of HSPG in ACC3 cells. Such a degradation pathway has been predicted morphologically (*20*), as described above. A similar degradation process was observed by Iozzo in a human colon carcinoma cell system in a pulse-chase experiment (*10*). Furthermore, he analyzed the process of degradation of [^{35}S]sulfate-labeled HSPG molecules in the same cell system (*29*), although no investigation has been performed on the core protein. In human endothelial cells (*30*), proteases, such as stromelysin or plasmin, as well as heparanases are needed to release bFGF from HSPG-bFGF conjugates. It is hence highly suggested that ACC3 cells are capable of degrading the core protein of HSPG by means of their own proteases in the course of cell growth. In fact, we have already confirmed that the core protein of HSPG was degraded by 2 μg/ml of stromelysin and 0.5 unit/ml of plasmin (unpublished data). It is important to elucidate the turnover of HSPG in ACC3 cells, because this information could be highly relevant as to the clinical mode of adenoid cystic carcinomas, such as frequent vascular invasion and hematogenous metastasis. We have suggested that such clinical characteristics might be caused by the affinity for basement membranes of adenoid cystic carcinoma cells based on the results of an immunohistochemical study (*3*). However, no molecular mechanism for these phenomena has been presented so far. More information is needed on the intracellular localization of HSPG in terms of the secretion and degradation pathways of the core protein and on proteolytic enzymes which are involved in the degradation.

The core protein of HSPG of ACC3 cells was clearly shown to contain *N*-linked oligosaccharides in addition to heparan sulfate chains. From the amino acid sequence (*12, 13*), ten potential sites for *N*-linked oligosaccharide attachment, Asn-X-Ser (Thr) sequences, are predicted in the core protein. However, the existence of *N*-linked oligosaccharides in the core protein has not been reported yet. The results of digestion with PNGase and Endo-H of the mature form of HSPG unequivocally indicated that the core protein

of HSPG contained Endo-H-resistant complex type oligosaccharide chains, which were processed from Endo-H-sensitive high-mannose ones. An interesting fact was that the HSPG molecules secreted in the medium had complex-type oligosaccharide chains. The processing to the complex type might be a critical event for the secretion of HSPG in this cell system. Similar results were obtained for E-selectin of human endothelial cells (*31*). However, the conversion to the complex type or maturation steps for *N*-glycosylation do not seem to be an essential requirement for cell surface expression of glycoproteins in some cell lines, such as hepatitis B virus envelope glycoprotein in MDCK cells (*32*) and asialoglycoprotein receptor in Hep G2 cells (*33*). However, there is no such information yet on proteoglycans. Further investigation will be needed on the mechanism of maturation of *N*-linked oligosaccharide chains, before we conclude whether the present results are critical evidence as to the secreting process of HSPG or not.

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