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Perlecan (heparan sulfate proteoglycan) gene expression reflected in the characteristic histological architecture of salivary adenoid cystic carcinoma

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Abstract In order to determine the role of the basement membrane-type heparan sulfate proteoglycan (HSPG), known as perlecan, in the formation of the characteristic cribriform structures of salivary adenoid cystic carcinomas, the mode of expression of mRNA for the core protein of HSPG was investigated by using in situ hybridization (ISH) both in surgical specimens and in a cell system (ACC3) of adenoid cystic carcinomas. In the surgical specimens, the mRNA for the HSPG core was more intensely expressed in solid tumor cell nests, especially in smaller ones. Within the nests, the signals were detected almost exclusively in cuboidal cells forming small pseudocysts. In contrast, signals were absent in flat cells forming large pseudocysts or in carcinoma cell nests attaching to the peripheral nerves or blood vessels. In normal salivary gland tissues, myoepithelial cells expressed the mRNA at a high level, but acinar and duct epithelial cells did not. In the time-course experiment of ACC3 cells, signals for HSPG core increased with time and reached the maximum on day 4, decreasing thereafter in a culture condition in which cells reached confluence in a week. The results indicate that HSPG is biosynthesized by adenoid cystic carcinoma cells which are in the proliferation phase, and that tumor cells producing HSPG tend to form initial structures of stromal pseudocysts.

Key words Adenoid cystic carcinoma · Basement membrane · Heparan sulfate proteoglycan · In situ hybridization · Perlecan

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Introduction

Basement membrane-type heparan sulfate proteoglycan (HSPG), in recent times known as perlecan, has been isolated and characterized from ACC3 cells, which are an established cell system from an adenoid cystic carcinoma of the human parotid gland [15]. Its core protein is 470 kDa in size, which is almost equal to the core proteins of other sources, such as mouse EHS tumors [10], bovine endothelial cells [22], and human colon carcinoma cells [11]. The primary structure of HSPG core protein was determined from human and mouse cDNA clones, and its biological functions have been suggested from its multidomain structure with possible attachment sites for heparan sulfate (HS) chains and oligosaccharides [14, 19]. However, functional roles of HSPG, especially in tumor cell activities, are still poorly understood. Recent studies have revealed that basement membrane molecules, including HSPG, are not only localized in the basement membrane but also distributed in the stromata of neoplastic or inflammatory tissues [17, 18, 20, 23]. These data imply that basement membrane molecules function in the proliferation of both tumor cells and stromal cells and in tissue remodeling during tumor invasion in addition to granulation tissue formation.

Adenoid cystic carcinomas arising in human salivary glands are histopathologically characterized by cribriform structures which result from stromal pseudocysts. The stromal space, especially pseudocystic spaces, has been shown immunohistochemically to be abundant in basement membrane molecules including HSPG [6]. These molecules have also been demonstrated to be synthesized by parenchymal cells of adenoid cystic carcinomas using ACC2 and ACC3 cells which were established from dependent adenoid cystic carcinomas of the human salivary gland [7]. When ACC2 cells were cultured in collagen gel, the cells formed pseudocystic cavities, which were rich in HSPG and other basement membrane molecules, within their spherical colonies [16]. Since the biosynthetic activity for HSPG was especially pronounced in ACC3 cells, we have also demonstrated its intracytoplasmic

pathway in ACC3 cells by immunoelectron microscopy [12]. These lines of evidence suggest that the characteristic histology of adenoid cystic carcinomas results from the overproduction of basement membrane molecules by tumor cells. Furthermore, we were able to show the close relationship between the growth of ACC3 cells and their biosynthesis of HSPG [15]. However, it is still unknown which cells of adenoid cystic carcinoma are responsible for the HSPG production in tissues or in which stage of pseudocyst formation it is produced. To this end, it is expected that ISH experiments will be performed.

In this study, we carried out ISH in tissue samples of adenoid cystic carcinoma and ACC3 cells by using RNA probes for HSPG core. The gene expression of HSPG core was enhanced in carcinoma cells in their proliferating foci/phases and was related to the initiation step in the formation of pseudocystic structures.

Materials and methods

Cells

The cell system, ACC3, was established from an adenoid cystic carcinoma arising in a human parotid gland as previously described [7]. The cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, Calif.), 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. 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, and cultivated for 8 days. The conditions were exactly the same as in the previous immunofluorescence, immunoprecipitation, and Northern blotting experiments [7, 15]. In these culture conditions, ACC3 cells reached confluence on day 7.

Tissue samples

Tissue samples of adenoid cystic carcinomas of human salivary gland were obtained from ten surgical specimens collected at the Department of Pathology, Faculty of Dentistry, Niigata University, in the 5 years from 1994 to 1998. All specimens were routinely fixed in 10% formalin and embedded in paraffin. Serial 5-µm-thick sections were cut from paraffin blocks. All procedures were carried out under treatment with diethylpyrocarbonate (DEPC) water to avoid RNA damaged by RNAse.

Preparation of RNA probes

HSPG RNA probes were prepared with a digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) using SP6/T7 RNA polymerase (Promega, Madison, Wis.) as previously described [15]. Briefly, template cDNA (1.5 kb, corresponding to domain III of human perlecan, provided by Dr. K. Tryggvason) [14] 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.

In situ hybridization

Two dishes each of ACC3 cells were fixed in 4% paraformaldehyde for 15 min every 48 h for 8 days. 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 subjected to two 10-min washes in PBS containing 5 mM $MGCL^2$, and then treated with 1 µg/ml of proteinase K in Tris-HCl buffer 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× saline sodium citrate (SSC), 50% formamide and 500 ng/ml of probe. After hybridization, the cover glasses were rinsed in 2× SSC and washed in 0.1 M maleic acid buffer (pH 7.5) containing 0.05% Triton X-100, after which the hybridized probes were detected with DIG detection kits (Boehringer Mannheim) using alkaline phosphatase-conjugated sheep anti-DIG antibodies. Color development was performed using BCIP/NBT as substrates for alkaline phosphatase at 37°C for 15 min. The cells on cover glasses were counterstained with methyl green and mounted on slide glasses.

Paraffin sections were dried at 60°C for 1 h and then deparaffinized with xylene and series of ethanol. After deparaffinization, sections were washed with standard saline citrate (SSC), followed by digestion with 5 µg/ml proteinase K (Sigma) at 37°C for 10 min. Sections were then washed with glycine buffer, dehydrated through an ascending ethanol series, acetylated with 95% ethanol/5% acetic anhydride solution at –20°C for 15 min, and hybridized as described above. After color development, they were counterstained with methyl green. Representative slides were photographed on Fujichrome 100 film.

Results

Time course of ISH in ACC3 cells

On day 2 after plating, weak and diffuse signals for HSPG core mRNA were observed mainly in the periphery of the cytoplasm of ACC3 cells (Fig. 1a). On day 4, when the cells were shown to synthesize HSPG core protein most actively [15], hybridization signals were accumulated all over the cytoplasm, indicating that the mRNA for HSPG core was highly expressed in the cells (Fig. 1c). On day 6, the cells spread widely with their cytoplasmic processes extended and came into contact each other, indicating that they were in a subconfluent status. The signals were definitely lower in intensity and population than those of day 4, and they were localized around vacuolar structures in the periphery of the cytoplasm (Fig. 1e). On day 8, when the cells had already reached confluence, the intensity and intracellular distribution of signals were basically the same as those of day 6 (Fig. 1g). This time course change of HSPG core mRNA signal levels resembled that shown by our previous Northern blotting analysis [15], in which the expression of mRNA for HSPG core was most markedly enhanced on day 4 in the same culture conditions for ACC3 cells as in the present experiments. The results indicated that the expression of HSPG core mRNA was in concordance with its protein synthesis in ACC3 cells, and that the growth of ACC3 cells corresponded with their HSPG biosynthetic levels. In control experiments by hybridization with sense probes, signals were scarcely detected in cells throughout the experimental period from days 2 to 8 (Fig. 1b, d, f, h).

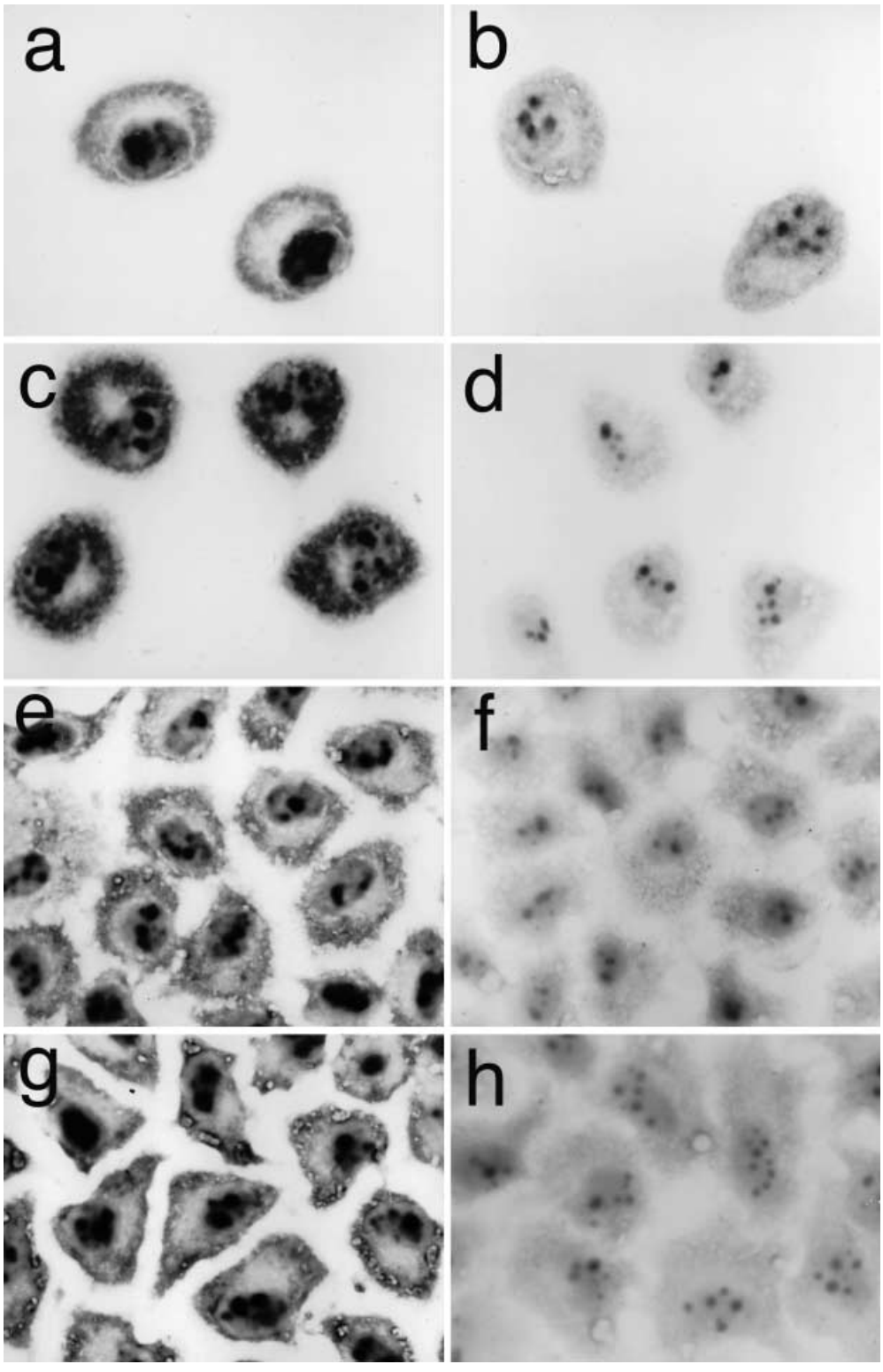
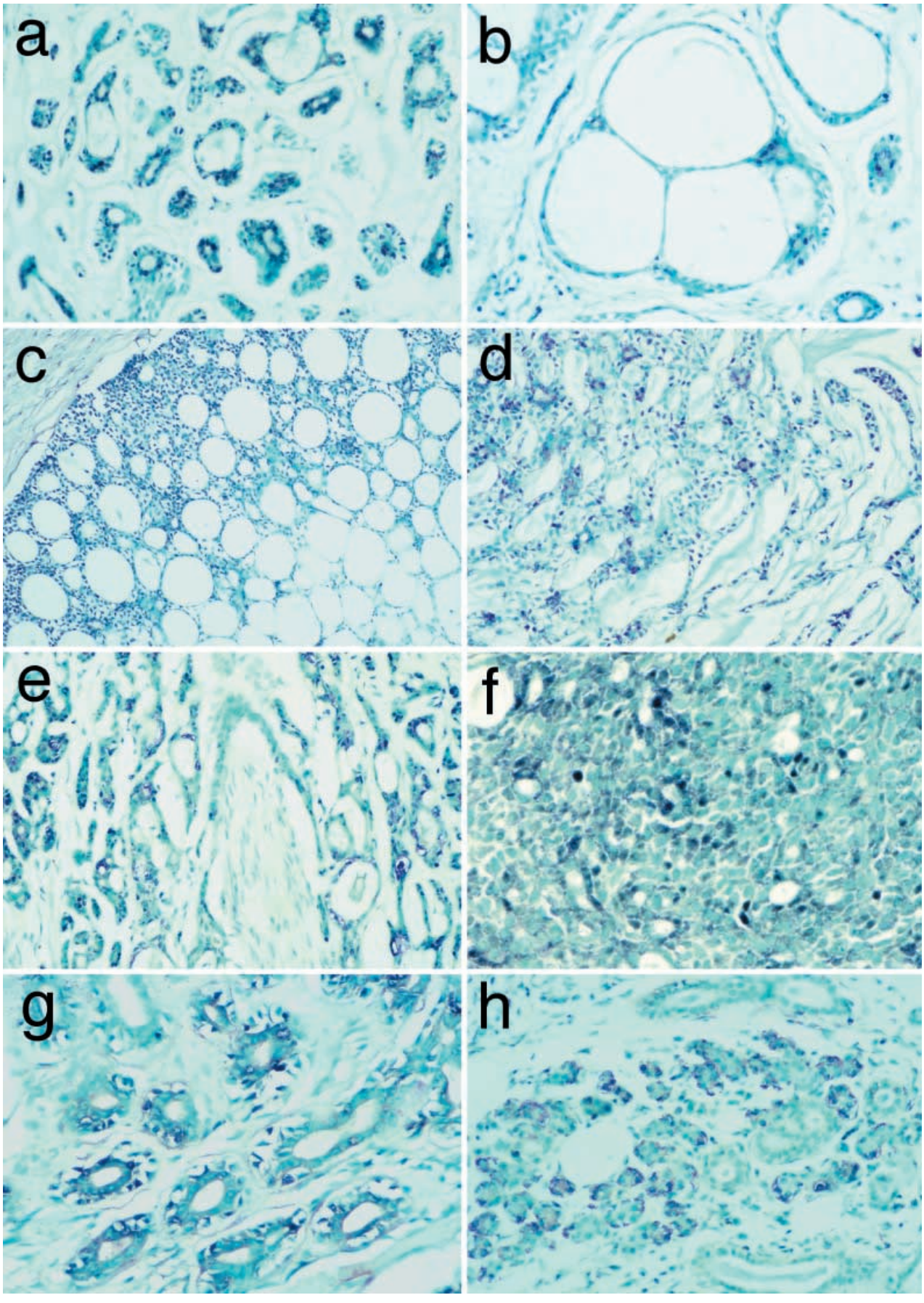


Fig. 1a–h In situ hybridization for basement membrane type heparan sulfate proteoglycan core protein mRNA in ACC3 cells. **a, b** day 2, **c, d** day 4, **e**, **f** day 6, **g, h** day 8. Signals were observed in cells hybridized with antisense probes (**a**, **c**, **e**, **g**), while none were in control cells hybridized with sense probes (**b**, **d**, **f**, **h**) ×400. Digoxigenin-immunoalkaline phosphatase, methyl green counterstain. The strongest signals were obtained on day 4

Fig. 2a–h In situ hybridization for basement membrane type heparan sulfate proteoglycan core protein mRNA in adenoid cystic carcinoma tissues. **a** Small invasive tumor cell nest. ×120 **b** Cribriform type. ×200 **c** Cribriform type. ×100 **d** Trabecular type. ×180 **e** Trabecular type. ×200 **f** Solid type. ×300 **g** Tubular type. ×200 **h** Normal salivary gland. ×160 Digoxigenin-immunoalkaline phosphatase, methyl green counterstain. Signals were detected in small tumor cell nests (**a–e**), especially in cuboidal cells forming small pseudocysts (**b–f**), while none were in flattened cells of large pseudocysts (**a–c**). Positive signals of tumor cells in trabeculae decrease definitely, when they attach to peripheral nerves (**e**). Signals in outer cells in tubular structures (**g**) resemble those in myoepithelial cells of acini or intercalated ducts in normal salivary gland (**h**) ▶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ISH in surgical specimens of adenoid cystic carcinoma

In tissue samples of adenoid cystic carcinomas, signals for HSPG core mRNA in tumor cells were detected irrespective of their histology of tumor cell nests. We classified tumor cell nests into four histological types, cribriform (Fig. 2a–c), trabecular (Fig. 2d–e), solid (Fig. 2f) and tubular (Fig. 2g), as in the previous study [6]. Stronger signals were detected in smaller tumor cell nests regardless of their histological types.

In the cribriform type foci, mRNA for HSPG core was highly expressed in cuboidal cells which were forming small pseudocysts but was not apparent in polygonal cells forming large pseudocysts (Fig. 2a). The expression was extremely suppressed in those in a monolayer of flattened cells which seemed to be stretched by an excessive retention of luminal contents of pseudocysts (Fig. 2b). In large foci of the cribriform type, tumor cells were densely packed in the peripheral area, but they were arranged in a reticular fashion owing to pseudocyst formations in the central area (Fig. 2c). Therefore, there was a general tendency for the signals to be localized in the periphery in tumor cell nests of this type.

In the trabecular type of foci, signals were detected along the slender cord of tumor cells but were most enhanced in cell foci, initiating the formation of small pseudocystic structures (Fig. 2d). It is well known that adenoid cystic carcinoma cells tend to invade along peripheral nervous fibers, vascular vessels and muscle fibers, which are abundant in basement membranes. In this ISH experiment, we could localize HSPG mRNA signals in the tumor cell nests invading the area around peripheral nerves. Stronger signals were detected in tumor cell strands surrounding neural tissues, while no positive signals were observed in tumor cell nests which were closely attached to the perineurium (Fig. 2e). This result suggested that production of HSPG was not necessary for adenoid cystic carcinoma cells on the perineurium because HSPG was plentifully assembled into the structure.

In the solid type of foci, signals were observed in cell foci forming small pseudocysts, although these pseudocystic structures were not so evident in the HE-stained section (Fig. 2f). However, there were no definite signals in the tumor cells, which were arranged evenly and densely.

In the tubular type of foci, in which duct-like structures were composed of two cell layers, strong signals were observed in the cells of the outer layer but only weak ones in the inner cells (Fig. 2g). This indicated that the outer cells, which are topographically myoepithelial and facing the extracellular milieu, were more capable of producing HSPG than the inner duct epithelial cells. A similar topographically differential expression for HSPG core mRNA was observed in normal salivary gland tissues which were included in the sections of adenoid cystic carcinomas. Strong signals for HSPG core mRNA were localized in myoepithelial cells of acini or intercalated ducts, but only weak signals were observed in acinar cells or duct epithelial cells (Fig. 2h).

Discussion

Previous immunohistochemical and immunocytochemical studies have shown that ACC3 cells, which were established from a human adenoid cystic carcinoma, produce basement membrane HSPG, or perlecan [7, 12, 16]. Furthermore, the HSPG produced by ACC3 cells was shown to be biochemically analogous to those isolated from other cell types [15]. The determination of the gene expression patterns of HSPG in tissues and cells of adenoid cystic carcinoma should be the next essential step in understanding the characteristic histological architecture and clinical and biological behavior of adenoid cystic carcinomas. In the present study, the mode of expression of HSPG core mRNA both in the ACC3 cells and in tissue samples of adenoid cystic carcinoma was determined by ISH for the first time.

The results of ISH for ACC3 cells clearly indicate that the expression of HSPG corresponded with the growth of ACC3 cells in culture. It is thus suggested that HSPG molecules were important for the their growth. This result may be consistent with our previous data obtained in time course experiments by metabolic labeling for HSPG in a near-identical culture condition as the present experiment [15]. In those experiments, HSPG biosynthesis increased rapidly until day 4 after plating, and its secretion and/or shedding became significant on days 6–8 after plating. Northern blotting analyses also showed a maximal increase of HSPG core mRNA per cell on day 4 and a decrease thereafter. Our previous immunohistochemical and immunocytochemical studies by confocal microscopy [7] and immunoelectron microscopy [12] have also suggested that the immunolocalization pattern of HSPG in ACC3 cells changed concomitantly with cell growth. Taken together, these findings indicate that the expression of HSPG in ACC3 cells is regulated by their cell growth, and that HSPG is important for controlling proliferation of ACC3 cells in culture.

Recently, Sharma et al. [26] reported that HSPG (perlecan) suppression inhibited tumor cell growth and angiogenesis in human colon carcinoma using antisense constructs. Similar results were obtained in melanoma cells [1].

Adenoid cystic carcinomas are histopathologically characterized by cribriform structures which are composed of pseudocysts. The pseudocysts contain HSPG and other basement membrane molecules within the lumina [3, 6, 9, 29, 30]. In the present experiment, tumor cells forming small pseudocysts expressed HSPG core mRNA prominently. However, the larger the pseudocysts grew, the lower the HSPG core mRNA expression became. The results indicate that adenoid cystic carcinoma cells in small nests in which they keep in contact with each other are able to synthesize HSPG and secrete and deposit it into the intercellular space to form pools of HSPG. When such a HSPG pool grows large enough to be recognizable as a pseudocystic space, carcinoma cells may cease to express the mRNA instead, but secreted HSPG molecules were retained in forms with which the

antibodies crossreacted. The expression of HSPG core mRNA may be suppressed when cell membrane receptors of tumor cells are saturated with signals from extracellular HSPG molecules. Therefore, the formation of pseudocysts is likely to be initiated by intercellular deposition of HSPG. This was also demonstrated in three-dimensional collagen gel culture of ACC2 cells of human adenoid cystic carcinoma origin [16]. In our previous immunohistochemical study, we speculated that tumor cell strands formed pseudocysts by their invagination or enclosure of the stromal space [6]. However, the present ISH result may indicate that our previous interpretation was just one of the ways this occurred. The elevated signals for HSPG core mRNA in pseudocystic structures in tumor cell nests of the solid or trabecular types would also support the idea that pseudocysts are generated by a focal intercellular pool of HSPG. The characteristic cribriform structures are therefore regarded as a terminal feature of enhanced intercellular deposition of HSPG and other ECM components. An extreme example for excessive growth of pseudocysts should be a reticular appearance of adenoid cystic carcinoma nests [6]. However, it is still controversial how tumor cells are polarized and synchronized for HSPG/ECM secretion to form pseudocysts and how sizes of pseudocysts are controlled in each tumor cell nest which is varied in cell density. To explain these events at the molecular levels, further experiments using the three-dimensional tissue culture system in which pseudocystic structures are formed in adenoid cystic carcinomas cell nests would be useful [16].

In the tubular type foci of adenoid cystic carcinomas, HSPG core mRNAs were definitely expressed more strongly in the outer cells of tubular structures. This result was interpreted as higher HSPG expression in myoepithelial cells. This was also demonstrated in the normal salivary gland tissue in the present study. Therefore, it is possible to say that myoepithelial cells located in the outer layer of tubular structures need to produce HSPG for a component of basement membranes by which they interface with their extracellular milieu. Since the ductal structures in adenoid cystic carcinomas should not function as ducts [2, 4, 5, 21], myoepithelial differentiation of the outer cells may be important just for its topographical phenotype. However, it cannot be expected that they will function as myoepithelial cells [4, 5]. We consider that the topographic location of tumor cells is rather important for their induction of HSPG expression. Since ACC3 cells have biphasic characters of differentiation to both duct epithelial and myoepithelial cells [12], the present result from their in vitro biosynthesis of HSPG may partly support the results from surgical specimens.

Adenoid cystic carcinomas are known frequently to invade the peripheral nervous tissue and result in metastases to the central nervous system via peripheral nerves after a long interval [8, 24, 27, 28]. In our previous study, we suggested that such a peculiar mode of invasion might be caused by an affinity of adenoid cystic carcinoma cells for the basement membrane [6]. We believe that this interpretation is now confirmed again by the present result, which demonstrates that the tumor cells attaching to the perineurium did not express HSPG core mRNA, while those of the outer trabeculae expressed a higher level. The results suggest that adenoid cystic carcinoma cells need HSPG for their growth. They produce HSPG in circumstances in which HSPG is poor, but no HSPG production is necessary in the peripheral nerves, where abundantly localized HSPG molecules control neural cell differentiation and growth [13, 25].

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