Perlecan-rich epithelial linings as a background of proliferative potentials of keratocystic odontogenic tumor

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BACKGROUND: The intraepithelial deposit of perlecan, a basement membrane-type heparan sulfate (HS) proteoglycan, has been demonstrated in neoplastic conditions such as salivary gland tumors, odontogenic tumors, and oral carcinoma in situ. Our aim was to determine whether perlecan turnover was enhanced in the lining cells of keratocystic odontogenic tumor (KCOT), which had been recently renamed from odontogenic keratocyst because of its accumulated evidence of neoplasm, as a possible background for neoplastic proliferation.

METHODS: Ten surgical specimens from each of KCOT, dentigerous cyst, and radicular cyst were examined for the expressions of perlecan core protein, HS chains, heparanase, and Ki-67 by immunohistochemistry and in situ hybridization.

RESULTS: In KCOT, perlecan core protein and HS chains were localized on the cell border from the parabasal to subkeratinized layers of the lining epithelium. Heparanase was localized in a similar fashion to those for perlecan and HS chains but was within the cytoplasm. mRNA signals for perlecan core protein and heparanase were mostly compatible with their protein signals. Ki-67-positive cells were localized mainly in the second basal cell layers with definitely higher labeling indices (approximately 31.3%, second layer). In contrast to KCOT, dentigerous cysts and radicular cysts had no perlecan, HS chains, and heparanase deposition in their linings with extremely lower Ki-67 indices (0.4–0.8%).

CONCLUSION: The result suggests that the characteristic intra-lining-epithelial deposit of perlecan in KCOT, which has never been seen in other cystic jaw lesions, is a new evidence supporting the neoplastic nature of KCOT.


Keywords: cystic lining epithelium; intraepithelial stroma; keratocystic odontogenic tumor; Ki-67 labeling index; perlecan

Introduction

Perlecan, a heparan sulfate proteoglycan (HSPG), is one of the constituent molecules of the basement membrane. However, it has been demonstrated in recent years that perlecan is distributed not only in the basement membranes, but also in the stromal space in various pathophysiologic conditions (1–4). Histopathologically, the stromal space where perlecan is accumulated has been characterized by myxoid appearances (1, 5, 6). More recently, widening of the epithelial intercellular space specifically found in ameloblastoma foci (7), tooth enamel organs (8), and squamous epithelial dysplasia and carcinoma in situ of the oral mucosa (9) has been shown to be due to perlecan deposition. It is thus considered that the myxoid appearance or widened intercellular space is caused by the deposition of perlecan where water molecules accumulate. Such perlecan-rich myxoid tissues are beneficial for cellular proliferation because perlecan contains bioactive domains in its core protein and growth factor reservoir-like HS chains (10).

Keratocystic odontogenic tumor (KCOT) has been classified as a developmental jaw cyst using the term odontogenic keratocyst (OKC) since it was first reported by Philipsen in 1956 (11). However, neoplastic characteristics in OKC have been suggested from clinical aspects (12–16) as well as from pathologic aspects (17, 18). Recently, the most convincing evidence for the neoplastic nature of OKC has been established: mutations of the patched-1 (PTCH) gene were first demonstrated in nevoid basal cell carcinoma syndrome (NBCCS) (19). Additionally, several PTCH gene mutations have been confirmed even in sporadic OKC cases (20–22). Based on these lines of evidence, the WHO 2005 (Philipsen) has proposed the neoplastic disease concept

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of OKC by introducing its new term of KCOT, a benign neoplasm (23).

The purpose of this study was to demonstrate the expression mode of perlecan, together with HS chains and its lyase, heparanase, at both protein and gene levels in the lining epithelium of KCOT to determine whether the perlecan-rich intraepithelial stroma is prepared for cellular proliferation in the KCOT lining as has been previously confirmed in oral carcinoma in situ or ameloblastoma.

Materials and methods

Materials

Ten surgical specimens from each of KCOTs, dentigerous cysts, and radicular cysts were collected for the present study from the surgical pathology files of the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences, during a 10-year period from 1997 to 2006 after histopathologic examinations of tissue sections stained with hematoxylin and eosin. The samples were fixed in 10% formalin and routinely embedded in paraffin. Serial sections cut at 4 μm from paraffin blocks were used for immunohistochemical stainings and in situ hybridization.

Antibodies

Antibodies against the mouse basement membrane-type perlecan core protein were raised in rabbits as described elsewhere (24). The antibodies have been shown to recognize human perlecan epitopes (7–10). A mouse monoclonal antibody against HS chains of human fetal lung fibroblasts (clone F58-10E4), which recognizes a native epitope in HS chains, was obtained from Seikagaku Corporation (Tokyo, Japan). Rabbit polyclonal antibodies against human heparanase were prepared as described previously (25). A mouse monoclonal antibody against Ki-67 of human origin (clone MIB-1) was obtained from DakoCytomation (Copenhagen, Denmark).

Immunohistochemistry

Paraffin sections were subjected to immunohistochemical stainings for perlecan core protein, HS chain, heparanase, and Ki-67 by using the Envision+/HRP system (DakoCytomation). For Ki-67 antigen retrieval, deparaffinized sections were autoclaved in citric acid buffer (pH 6.0), at 120°C for 10 min. After autoclave treatments, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) containing 0.5% skimmed milk and 0.05% Triton X-100 (T-PBS) and treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activities. After rinsing in T-PBS, they were incubated with 5% skimmed milk in T-PBS for 1 h at room temperature to block non-specific protein-binding sites. They were then incubated overnight at 4°C with the primary antibodies diluted at 50 μg/ml in T-PBS. After incubation, the sections were rinsed in T-PBS and incubated with the secondary antibodies (anti-rabbit or antimouse immunoglobulins) which were conjugated with peroxidase-labeled dextran polymers for 1 h at room temperature. After rinsing with T-PBS, they were treated with 0.02% 3,3′-diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide to visualize the reaction products. Finally, the sections were counterstained with hematoxylin. For control studies on antibodies, the primary antibodies were replaced with pre-immune rabbit IgG or mouse IgG subclasses (DakoCytomation).

Ki-67 labeling indices

The percentage of lining epithelial cells with Ki-67-positive nuclei among 100 cells in each epithelial layer was calculated in four fields, and the mean values were determined as Ki-67 labeling indices.

Preparation of RNA probes

The domain I region of human perlecan cDNA was amplified by RT-PCR using RNA samples of normal oral mucosa and 5′-CTGCT GCCGG TGACC CAT GG-3′ (#135 to #154, sense) and 5′-TTGGG AACTG GGGCA CTGTG-3′ (#651 to #669, antisense) primers, followed by nested-PCR using 5′-CGGGA TCCGT GACCC ATGGG CGTC-3′ (#144 to #171, sense), and 5′-CCCAA GCTTG GGAC TGTGC CCAGG CGTC-3′ (#633 to #659, antisense) primers. The PCR products (516 bp) were subcloned into plasmid vector (pBluescript II, Promega Corporation, Madison, WI, USA), and were digested with BamHI and HindIII. The linearized plasmids were used as templates to synthesize digoxigenin-labeled RNA antisense probes by T7 RNA polymerase (Promega) and sense probes by T3 RNA polymerase (Promega) (9).

Human heparanase cDNA was amplified by RT-PCR using RNA samples of normal oral mucosa and 5′-TGGAC CTGGA CTTCT CCACC-3′ (#116 to #135, sense) and 5′-TGTAT TCCTT CTTGG GAT CG-3′ (#312 to #331, antisense) primers. The PCR products (216 bp) were subcloned into plasmid vector (pGEM-T Easy vector, Promega Corporation), and were digested with NcoI and SalI. The linearized plasmids were used as templates to synthesize digoxigenin-labeled RNA antisense probes by T7 RNA polymerase (Promega) and sense probes by SP6 RNA polymerase (Promega).

In situ hybridization

Paraffin sections were cut at 5 μm. After deparaffinization, sections were washed in three changes of 2X standard saline citrate (SSC) and treated with 5 μg/ml of protease K (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 20 min at 37°C. They were then washed with 0.2% glycine in PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) for 5 min dehydrated with a series of ethanol (70–100%), and air dried. Hybridization was performed for 15 h at 48°C in a moist chamber. The hybridization solution contained 10% dextran sulfate, 1x Denhardt’s solution, 100 μg/ml of salmon sperm DNA, 125 μg/ml of yeast tRNA, 3x SSC, 50% formamide, and 500 ng/ml of probes in 10 mM phosphate buffer (pH 7.4). After hybridization,
Results

Immunohistochemical localizations for perlecan, HS chain, heparanase, and Ki-67 were compared among KCOTs, dentigerous cysts, and radicular cysts. As to perlecan and heparanase, their gene expression modes were also determined by in situ hybridization. The results are described separately below for each cystic lesion. Immunopositive ratios among the examined cases for these antigens and Ki-67 labeling indices by three epithelial zones, the first and second basal layers and the third layer and higher, were compared between the three cystic lesions and are summarized in Table 1.

Keratocystic odontogenic tumor

In the cyst lining of KCOT (Fig. 1a), perlecan was strongly immunolocalized mainly on the cell border of squamous epithelial cells from the second basal (parabasal) layer to higher levels and weakly in the basolateral border of basal cells including the epithelial basement membrane (Fig. 1b). Perlecan core protein was deposited between intercellular bridges occasionally forming round-shaped bubble-like deposits (Fig. 1c). mRNA signals for perlecan core protein were mostly compatible with their protein signals (Fig. 1d). The immunolocalization for HS chains was also basically the same as that for perlecan core protein but was more enhanced irregularly in the middle zone of the epithelial layer (Fig. 1e). It was thus confirmed that perlecan, a HSPG was produced by the lining epithelial cells and was deposited in their intercellular space.

Heparanase, which functions in the turnover of HS chains, was immunolocalized in a similar fashion as those for perlecan and HS chains but was within the cytoplasm. Within the cytoplasm, heparanase was obviously localized in the periphery, which was most enhanced in the basal cells showing its subnuclear localization (Fig. 1f). mRNA signals for heparanase were almost the same as its protein signals including their intracytoplasmic distribution (Fig. 1g). Ki-67-positive cells, which were in their cell cycle, were mainly localized in the second to third parabasal cell layers but occasionally in the basal cells (Fig. 1h). When Ki-67 labeling frequencies among epithelial cells were compared between epithelial layers from the first, second, and third and higher, those in the second basal layer showed the highest index (31.3%), followed by those of the third (11.1%) and the first (4.1%) basal layers (Table 1). The Ki-67 labeling indices of KCOT lining layers were extremely higher than those of other cystic lesions.

Dentigerous cyst

The lining epithelium of dentigerous cysts (Fig. 2a) showed no obvious immunopositivities for perlecan (Fig. 2b), HS chains (Fig. 2c), and heparanase (Fig. 2d). Ki-67-positive cells were not so frequently found in the lining epithelium (Fig. 2e), although a very small number of Ki-67-positive cells were scattered in the basal or parabasal layers. The labeling index of these cells was the highest in the basal first layer (1.5%), which was different from that of KCOT. It was, however, difficult to compare those values because the Ki-67 labeling indices for dentigerous cysts were extremely smaller than those of KCOT (Table 1).

Radicular cyst

The lining epithelium of radicular cysts (Fig. 3a) showed no immunopositive reactions for perlecan (Fig. 3b), HS chains (Fig. 3c), and heparanase (Fig. 3d). However, Ki-67-positive cells were occasionally localized only in the basal cell layer (Fig. 3e). Its labeling index was the highest in the basal first layer (2.9%), and this tendency was different from that of KCOT as mentioned above, although it was also difficult to compare those values because the actual labeling indices for radicular cysts were extremely smaller than those of KCOT (Table 1).

Discussion

In the present study, we have demonstrated for the first time the definite intercellular deposition of perlecan within the lining epithelium of KCOT. The presence of perlecan mRNA signals in the lining epithelium indicates that these epithelial cells are responsible for the production as well as the intercellular deposition of perlecan. The localization for HS chains and their lyase, heparanase, at both protein and gene levels, was also

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<tr>
<th>Cyst type</th>
<th>Lining epithelial layers</th>
<th>Immunopositivities (%) for</th>
<th>Ki-67 labeling indices (%) mean ± SD</th>
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<tr>
<td></td>
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<td>Perlecan</td>
<td>HS chain</td>
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<tr>
<td>Keratocystic odontogenic tumor</td>
<td>3rd+</td>
<td>100.0</td>
<td>100.0</td>
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<td></td>
<td>2nd</td>
<td>100.0</td>
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<td>Basal/1st</td>
<td>20.0</td>
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<td>Dentigerous cyst</td>
<td>3rd+</td>
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<td>Radicular cyst</td>
<td>3rd+</td>
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related to that of perlecan core protein. The results are basically the same as the enhanced expression levels of perlecan in neoplastic conditions of such squamous epithelial tissues as oral dysplasia and carcinoma in situ (9) and ameloblastoma (7), although the intercellular space of the keratocystic lining was not as wide as those neoplasms. In contrast to KCOT, dentigerous cysts as developmental cysts and radicular cysts as inflammatory cysts had no perlecan deposition and obviously less Ki-67 labeling indices in their lining epithelia, suggesting that KCOT was different in terms of lining cellular proliferation from the other types of jaw cysts.

Since the first report on the tissue, by Philipsen in 1956 (11), KCOT has been classified as one of the developmental jaw cysts by the term of OKC. However, there have been some reports suggesting neoplastic characteristics in OKC. For instance, clinically, it not only recurs frequently (12–15), but also develops into malignancies like squamous cell carcinomas (12–17). Histopathologically, its extraordinary recurrence rate has been explained by the presence of daughter cysts or epithelial islands in the connective tissue wall and dysplastic or neoplastic transformation (12, 15, 26) of lining cells, which have basically high proliferative

Figure 1 Histochemical profiles of keratocystic odontogenic tumor (KCOT). (a) Hematoxylin and eosin stain; (b and c) immunoperoxidase stain for perlecan core protein, hematoxylin counterstain; (d) in situ hybridization for perlecan core protein mRNA, digoxigenin-immunoalkaline phosphatase (DIG-ALP), methyl green counterstain; (e) immunoperoxidase stain for heparan sulfate (HS) chains, hematoxylin counterstain; (f) immunoperoxidase stain for heparanase; hematoxylin counterstain; (g) in situ hybridization for heparanase mRNA, digoxigenin-immunoalkaline phosphatase (DIG-ALP), methyl green counterstain; (h) immunoperoxidase stain for Ki-67, hematoxylin counterstain. (a, b, d–h) ×150; (c) ×600. Scale bars, 100 μm. In the linings of KCOT (a), perlecan core protein was localized strongly in the cell border from the parabasal to middle layers and weakly in the basolateral border of basal cells (b). Higher magnification of the lining epithelium indicates that perlecan core protein was deposited between intercellular bridges occasionally forming round-shaped bubble-like deposits (c). The localization of perlecan core protein mRNA signals was mostly compatible with the immunolocalization for perlecan core protein (d). HS chains were irregularly enhanced in the cell border from the parabasal to middle layer (e). Heparanase was localized in the parabasal to middle layers as well as in the basolateral border of basal cells (f). The localization for heparanase mRNA signals was almost the same as the immunolocalization for perlecan core protein (g). Ki-67-positive cells were only localized in the second to third parabasal cell layers but scarcely in the basal cells (h).
activity (27–31). However, the most convincing evidence for the neoplastic nature of OKC is the *patched-1* gene mutation first demonstrated in NBCCS (19). Since then, there has been a significant amount of accumulated evidence for the *patched-1* gene mutation not only in OKCs of NBCCS patients (32–35), but also in sporadic

Figure 2  Histochemical profiles of dentigerous cyst. Hematoxylin and eosin stain (a) and immunoperoxidase stains for perlecan core protein (b), heparan sulfate (HS) chains (c), heparanase (d), and Ki-67 (e), hematoxylin counterstain. ×150. Scale bar, 100 μm. The lining epithelium of dentigerous cyst (a) had neither immunopositivity for perlecan core protein (b), HS chains (c), nor heparanase (d). Ki-67-positive cells were scarcely found (e).

Figure 3  Histochemical profiles of radicular cyst. Hematoxylin and eosin stain (a) and immunoperoxidase stain for perlecan core protein (b), heparan sulfate (HS) chains (c), heparanase (d), and Ki-67 (e), hematoxylin counterstain. (a–d) ×70; (e) ×150. Scale bars, 200 μm. The lining epithelium of radicular cyst (a) had neither immunopositivity for perlecan core protein (b), HS chains (c), nor heparanase (d). In contrast, Ki-67-positive cells were occasionally found in the basal or parabasal cell layer (e). Perlecan was enhanced in the subepithelial connective tissue (b), and HS chains were also labeled in the vascular endothelial cells (c).
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OKCs (20–22). Eventually, in the WHO 2005 classification, OKC was renamed as KNOT and defined as a benign neoplasm (23). Our present results support the neoplastic character of KNOT from the point of view of the tumor-specific metabolism of perlecan, an extracellular matrix molecule.

From the present result demonstrating that the gene and protein expressions of perlecan were simultaneous, the high expression level of perlecan is maintained in the lining of KNOT. Therefore, the perlecan turnover from protein biosynthesis to HS chain degradation seems to be required to maintain its flat and constantly thin shape as well as its keratinization tendency of the lining epithelium of KNOT. As shown by our Ki-67 immunohistochemistry, the proliferating center of the lining epithelium of KNOT was located in the second basal layer. There have been some investigations on Ki-67 expression in OKCs (30, 36), but this characteristic positioning of Ki-67-positive cells in the second layer has never been intentionally investigated. Similar phenomena have already been shown in epithelial hyperplasia, epithelial dysplasia, and carcinoma in situ of the oral mucosa (37). The proliferating center of the oral epithelium is in the second basal (parabasal) layer in normal and hyperplastic conditions, but it extends into the first basal as well as into the upper layers in neoplastic conditions. The distribution of Ki-67-positive cells in KNOT was not as extensive as that of oral carcinoma in situ, and the lining cell proliferation might not be so active as a neoplastic condition.

A similar tendency was shown in the immunolocalization of perlecan which was mostly in the intercellular space, while intracellular localization of perlecan, which indicates accelerated biosynthesis, was observed in addition to the intercellular deposit in oral carcinoma in situ (9). In contrast to the perlecan deposition restricted to tumor parenchymal cells in intraepithelial neoplasms, it dramatically switches into the stromal space in invasive carcinomas (9). The intraepithelial deposition of perlecan has been confirmed in other epithelial neoplasms, such as ameloblastoma (7), adenomatoid odontogenic tumor (38), pleomorphic adenoma (5), and adenoid cystic carcinoma (6). These lines of evidence suggest that intraepithelial accumulation of perlecan is a requirement for cellular proliferation, which has been actually demonstrated in vitro (10).

Although the function of perlecan still remains poorly understood, that of the function of HS chains has been characterized as a reservoir for several kinds of growth factors, such as basic FGF (FGF2), FGF7, or TGF-beta (1–4). Another possible function would be its nutrient transport by diffusion through water molecules which accumulate around HS chains. These may benefit the proliferation and differentiation of the lining cells of KNOT. Moreover, domain V of the perlecan core protein contains laminin A chain-like repeats and EGF motifs (39), and intercellular accumulation of perlecan with EGF motifs may act directly as cell proliferating signals for the lining cells. HS chains are cleaved by heparanase, a lysosomal enzyme, at only restricted sites within the molecule, degraded into appreciable sized fragments within phagosomes as bioactive HS oligo-fragments (40). Heparanase has been shown to play an important role in invasion and metastasis of several kinds of tumors (40), such as breast carcinoma (41), malignant melanoma (42), and esophageal carcinomas (43). These results including our own indicate that the HS chain and perlecan turnover is one of the neoplastic settings.

From the present study, it is obvious that perlecan-rich milieu plays an important role in proliferation of lining cells and in growth of KNOT. However, neoplastic features have not necessarily been confirmed in any cases of KNOT. We have reported a KNOT case involving the maxillary sinus, in which the cyst lining showed ciliated cell metaplasia (44). It is, therefore, necessary to elucidate the whole metabolic pathways of perlecan including regulating mechanisms of the perlecan gene expression before complete understanding of the actual significance of perlecan expression in KNOTs is known.

References


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