J Oral Pathol Med (2004) 33: 87–95

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Intraepithelial expression of perlecan, a basement membrane-type heparan sulfate proteoglycan reflects dysplastic changes of the oral mucosal epithelium

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BACKGROUND: Intercellular deposition of perlecan, a major heparan sulfate proteoglycan (HSPG) of the basement membrane, is known to result in characteristic stellate reticulum-like structures in ameloblastomas or tooth germs. Although enlargement of the intercellular space is one of the histological characteristics of epithelial dysplasia of oral mucosa, the mode of expression of perlecan is poorly understood in these epithelial lesions.
METHODS: Eighty-two biopsy specimens consisting of normal and hyperplastic epithelium, epithelial dysplasia, and squamous cell carcinomas were examined for both perlecan core protein and heparan sulfate (HS) chains by immunohistochemistry and *in situ* hybridization.
RESULTS: In normal and hyperplastic epithelium, perlecan core protein and HS chains were localized in the cell border of parabasal cells and lower prickle cells, and HS chains were also found in basal cells. With an increase in the severity of epithelial dysplasia, the core protein was heavily and extensively deposited in the interepithelial space as well as in the cytoplasm of epithelial cells from the basal to the surface layers. Its gene expression was confirmed in the cells around the protein deposits. On the other hand, HS chains were enhanced in mild dysplasia, but decreased in moderate and severe dysplasias. In squamous cell carcinomas, either the core protein or HS chains were found scarcely in tumor cells but abundantly in the stromal space.
CONCLUSIONS: The findings indicate that perlecan is localized in the intercellular space of the oral epithelia, and that it is over-expressed in dysplastic epithelial cells and is deposited in their interepithelial space, which results in the histology of reduction of cellular cohesion.
J Oral Pathol Med (2004) **33**: 87–95

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Accepted for publication May 8, 2003

Keywords: basement membrane-type heparan sulfate proteoglycan; epithelial dysplasia; oral mucosa; perlecan; squamous cell carcinoma

Perlecan, a heparan sulfate proteoglycan (HSPG), is one of the major basement membrane macromolecules. However, it has been demonstrated in recent years that perlecan is distributed not only in the basement membranes but also in the stromal space of various pathophysiological conditions (1, 2). Histopathologically, such stromal spaces, which contain plentiful deposits of perlecan, are characterized by a myxoid appearance (3, 4). Both parenchymal cells and stromal cells seem to be active in proliferation in the myxoid tissue because such tissues with myxoid appearances are inevitably associated with immature granulation tissues (5–8) or with invasion fronts or proliferation foci of neoplastic tissues (8–10). More recently, perlecan has been shown to be rich in epithelial tissues, such as enamel organs of the tooth germ and ameloblastomas, in which intercellular spaces are prominent (11). It is thus considered that the myxoid appearance is caused by the deposition of perlecan where water molecules have accumulated, and that such perlecan-rich myxoid tissues have some advantage for cellular proliferation.

One of the 13 major histological characteristics of the oral epithelial dysplasia listed in the WHO Histological Typing of Cancer and Precancer of the Oral Mucosa (12), which had been originally proposed by the WHO Collaborating Centre for Oral Precancerous Lesions (13) is ''reduction of cellular cohesion,'' which was thought to be able to make epithelial cells behave autonomously. However, the reduction of cellular cohesion, in other words the enlargement of the intercellular space, has neither been of concern pathologically nor been investigated to a great extent. From the point of view that the enlarged intercellular space results from accumulation of proteoglycans, it is of great interest to investigate what is accumulated in the widened intercellular space of oral epithelial dysplasias.

In the present study, we studied expression of perlecan at both the protein and the gene levels by

immunohistochemistry as well as by *in situ* hybridization, together with the immunolocalization of HS chains in different conditions of the oral mucosal epithelium: from normal or hyperplastic epithelia to epithelial dysplasia or squamous cell carcinomas. We wanted to examine first whether perlecan is localized in the squamous epithelium of the oral mucosa, and if so, second, whether the mode of expression of perlecan is related to any dysplastic changes of the oral epithelium, and third, if perlecan deposition is related to any epithelial proliferation.

Materials and methods

Materials

Eighty-two biopsy specimens from the oral mucosa were selected from the surgical pathology files in the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences, during a 9-year period from 1992 to 2001. They consisted of 10 cases of normal epithelium, 10 of hyperplasia, 38 of epithelial dysplasia (mild, 22; moderate, 8; severe, 8), and 24 of squamous cell carcinomas (well-differentiated, 18; poorly differentiated, 6). Intraoral sites of the specimens taken were as follows: gingiva, 27; tongue, 22; hard palate, 3; buccal mucosa, 14; soft palate, 5; oral floor, 9. All the specimens were routinely fixed in 10% formalin and embedded in paraffin. Serial 5-μm sections were cut from paraffin blocks. One set of the sections was stained with hematoxylin and eosin and was used for re-evaluation of histological diagnosis, and the other sets were used for immunohistochemistry and *in situ* hybridization.

Antibodies

Antibodies against the mouse basement membrane-type HSPG (perlecan) core protein were raised in rabbits as described elsewhere (14). A mouse monoclonal antibody against HS chains of human fetal lung fibroblasts (clone F58–10E4), which recognizes a native epitope in heparan sulfate (HS) chains, was obtained from Seikagaku Corporation (Tokyo, Japan).

Immunohistochemistry

Paraffin sections were subjected to immunohistochemical stainings for perlecan core protein and HS chains by using the Envision+/HRP system (Dako Japan Co., Ltd., Kyoto, Japan). The sections were deparaffinized, rinsed in 0.01 M phosphate buffered saline (PBS, pH 7.4), and treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activities. After rinsing in PBS containing 0.5% skimmed milk and 0.05% Triton X-100 (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 (anti-core protein) or at 1:100 (anti-HS) in T-PBS. After incubation, the sections were rinsed in T-PBS and incubated with the secondary antibodies (anti-rabbit or anti-mouse immunoglobulins) that were conjugated with peroxidase-labeled dextran polymers for 30 min at room temperature. After rinsing with T-PBS, they were treated with 0.02% 3,3′-diaminobenzimine in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide to visualize reaction products. Finally, the sections were counterstained with hematoxylin. For control studies, non-immune rabbit or mouse IgGs were used instead of the specific primary antibodies. To demonstrate basement membranes of the mucosal epithelium, the sections were treated with 0.4% (w/v) pepsin (Sigma Chemical Co., St. Louis, MO, USA) in 0.01N HCl or 0.2% (w/v) trypsin (Sigma, type II) in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.1% $CaCl_2$ for 30 min at 37°C, prior to the incubation with the primary antibodies.

Preparation of RNA probe

The domain I region of human perlecan cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using RNA samples of normal oral mucosa and 5′-CTGCT GGCGG TGACC CATGG-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 CTGAG GGC-3′ (#144 to #171, sense), and 5′-CCCAA GCTTG GGCAC TGTGC CCAGG CGTC-3′ (#633 to #659, antisense) primers. The PCR products (516 bp) were subcloned into plasmid vectors (pBluescript II, Promega Corporation, Madison, WI, USA), and were digested with *Bam*HI and *Hin*dIII. 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).

In situ *hybridization*

In situ hybridization was performed as described previously (6, 8). In short, paraffin sections cut at 5 μm were deparaffinized, washed in three changes of 2× standard saline citrate (SSC), and treated with 5 μg/ml of proteinase K (Sigma) 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 30 min, dehydrated with a series of ethanol (70–100%), 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 probes. After hybridization, the sections were rinsed in 2× SSC and then the hybridized probes were detected with DIG detection kits (alkaline phosphatase, Boehringer-Mannheim GmbH, Darmstadt, Germany). The sections were counterstained with methyl green.

Results

Normal and hyperplastic epithelia

In normal epithelium (Fig. 1A), perlecan core protein was only faintly immunolocalized on the cell border of the parabasal cells (Fig. 1B), while HS chains were localized on the cell border of the basal cells in addition to on the cell border of the parabasal to lower prickle cell layers with an increasing tendency of their staining intensity towards the upper layer (Fig. 1C). mRNA signals for perlecan core protein were faintly detected in the suprabasal and lower prickle cells (Fig. 1D). In the lamina propriae and the

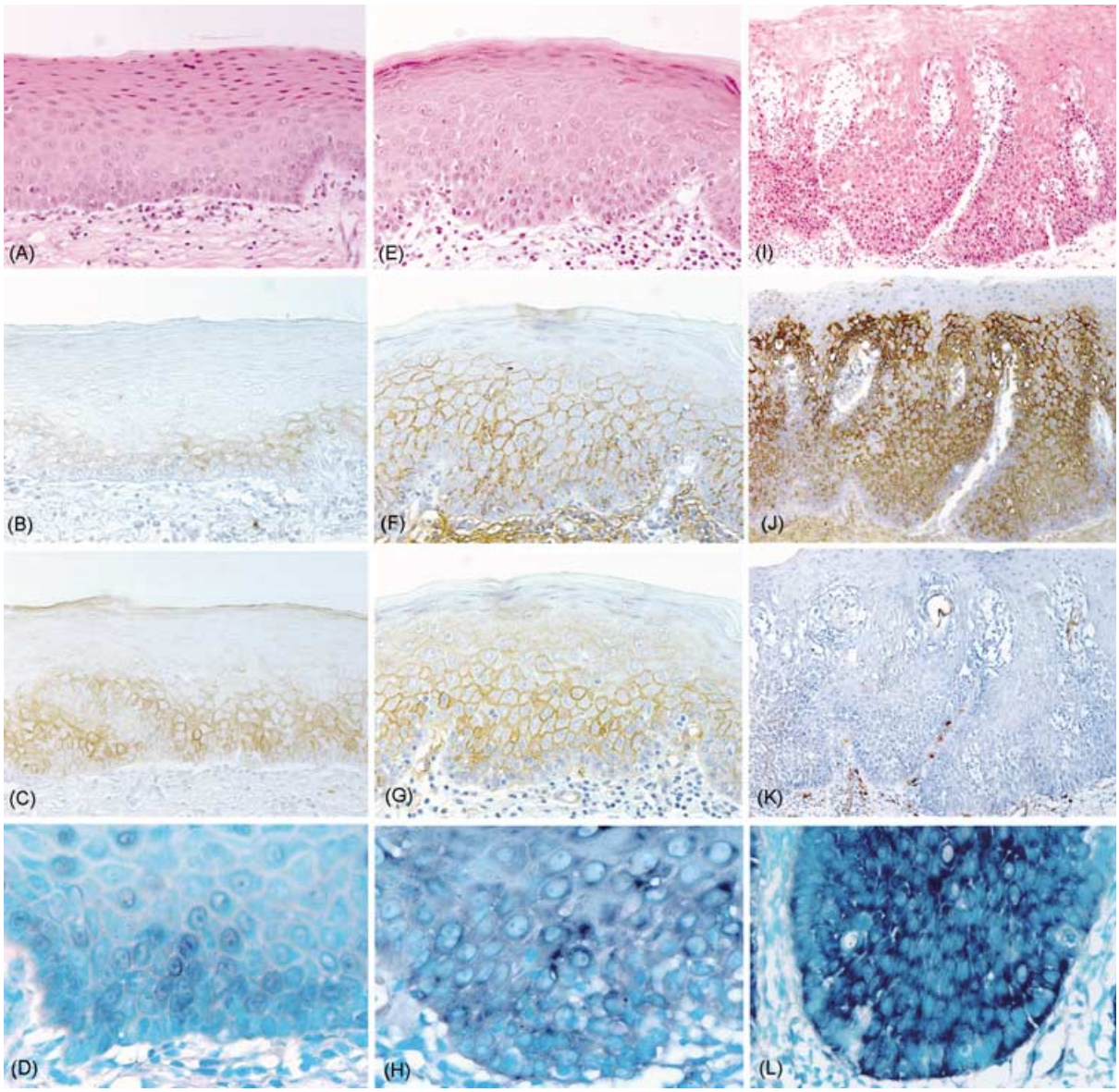

Figure 1 Normal and dysplastic epithelium of oral mucosa. (A, E, I) HE stain, upper layer; (B, F, J) immunoperoxidase for perlecan core protein, second layer, hematoxylin counterstain; (C, G, K) immunoperoxidase for heparan sulfate (HS) chains, third layer, hematoxylin counterstain; (D, H, L) *in situ* hybridization for perlecan core protein mRNA, lower layer, digoxigenin–immunoalkaline phosphatase (DIG–ALP), methyl green counterstain. (A–D) Normal, left column; (E–H) mild dysplasia, middle column; (I–L) moderate dysplasia, right column. A–C, E–G: ×200; I–K: ×100; D, H, L: ×300. In normal epithelium (A–D), perlecan core protein and HS chains were localized in the cell border of parabasal cells and lower prickle cells. Basal cells were only positive for HS chains but not for perlecan core protein. Perlecan core protein mRNA signals were faintly observed in epithelial cells in the lower layer (Fig. 1D). In mild epithelial dysplasia (E–H), expression modes for perlecan core protein and HS chains in the epithelium are basically the same as those for normal epithelium, although they were more clearly observed because of the expansion of the prickle cell layer. mRNA signals for perlecan core protein were almost compatible. In moderate epithelial dysplasia (I–L), the characteristic linear immunolocalization of perlecan on the cell border was enhanced and sometimes irregularly interrupted or coarsely punctuated with a diffuse intracytoplasmic staining. In contrast, no obvious immunolocalization for HS chains was seen in the prickle cell layer. mRNA signals for perlecan core protein were detected in the basal, parabasal, and prickle cell layers, which was basically the same as the distribution of perlecan core protein.

submucosal layer, perlecan core protein was localized in basement membranes of blood vessels and peripheral nerve fibers and was diffusely demonstrated in the myxoid connective tissue. The immunolocalization modes for perlecan and HS chains and mRNA messages for perlecan core protein in hyperplastic epithelia were basically the same as those in normal epithelia, although the staining intensities in hyperplastic epithelia were stronger (not shown).

Although the basement membrane of the oral mucosal epithelium was not demonstrated by the anti-perlecan core protein antibodies without any enzymatic pre-treatments, it was clearly demonstrated when the sections were treated with trypsin (Fig. 2A) or pepsin (Fig. 2B). When treated with

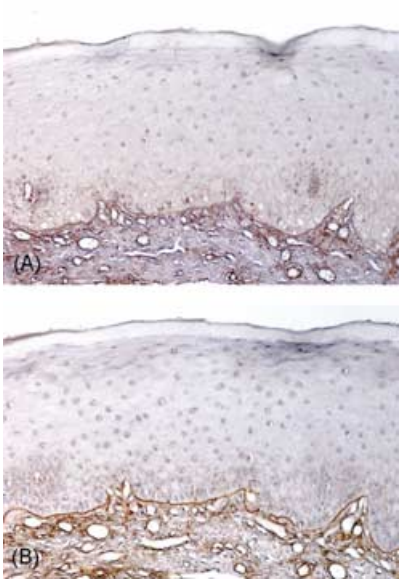

Figure 2 The differential staining patterns of perlecan core protein with enzyme treatments. (A) Pepsin-pre-treated paraffin section, (B) trypsin-pre-treated paraffin section. Immunoperoxidase, hematoxylin counterstain, ×125. In both sections, positive immunoreactivities for perlecan core protein in the basement membrane of normal epithelia were definitely seen (compare with Fig. 1(B) with no enzymatic pre-treatment). In contrast, the staining of intercellular space of epithelium was reduced.

either of these two proteinases, however, intraepithelial staining on the cell border disappeared or became weaker, while the stainings in other basement membranes were preserved.

Epithelial dysplasia

In mild epithelial dysplasia (Fig. 1E), perlecan core protein was more definitely immunolocalized on the cell border of the basal to prickle cells, showing a honeycomb-like appearance (Fig. 1F). There was no cytoplasmic staining, although the cell border staining was occasionally revealed by cross-cutting. The staining pattern was almost the same as that for HS chains (Fig. 1G). The staining intensities for both core protein and HS chains were obviously stronger than those in normal or hyperplastic epithelia. mRNA signals for perlecan core protein were also extended from the parabasal cell layer to both the basal and the prickle cell layers (Fig. 1H).

The staining intensities for perlecan core protein as well as its gene messages were enhanced with the severity of epithelial dysplasia from moderate to severe. In moderate epithelial dysplasia (Fig. 1I), the immunolocalization of perlecan core protein was extended to the upper prickle cell layer, and most of the epithelial cells other than surface-keratinized cells showed positive stainings (Fig. 1J). The linear immunolocalization of perlecan on the cell border, which resulted in a smooth honeycomb-like appearance, was sometimes irregularly interrupted and changed into a coarse-punctuated appearance. In addition to these cell border deposits, there was focally a diffuse intracytoplasmic staining for perlecan. In contrast, most of the honeycomb-like immunolocalization for HS chains disappeared from the prickle cell layer with an increase of the severity of dysplasia, except for irregular patched deposits in the cytoplasm, although the staining intensity of these cytoplasmic stainings varied from case-to-case (Fig. 1K). mRNA signals for perlecan core protein were detected in the basal, parabasal, and prickle cell layers, which was basically the same as the distribution of perlecan core protein (Fig. 1L).

In severe epithelial dysplasia in which basaloid cells proliferated monotonously replacing the epithelial layer (Fig. 3A), almost the whole layer of the epithelium, except for surface-keratinized cells, was positive for perlecan. Its immunolocalization was basically on the cell borders but occasionally within the cytoplasm of the basaloid cells, and sometimes the cell border and cytoplasmic stainings were merged with each other to form deeply stained coalescence, and the characteristic honeycomb-like appearance, which was seen in mild-to-moderate dysplasia, disappeared (Fig. 3B). HS chains were not discernibly immunolocalized in the prickle cell layer, which was basically the same as that in moderate epithelial dysplasia (Fig. 1K). However, irregular cytoplasmic as well as intercellular stainings for HS chains were occasionally observed in the upper epithelial layer (Fig. 3C). mRNA signals for perlecan core protein were intensely demonstrated in the whole epithelial layer as shown in a fashion similar to that for its core protein (Fig. 3D).

Squamous cell carcinoma

In well-differentiated squamous cell carcinomas (Fig. 3E), perlecan core protein was irregularly immunolocalized both in tumor cell nests and in myxoid stromata, which directly surrounded the tumor cell nests (Fig. 3F). Within the tumor cell nests, perlecan was randomly observed on the cell border or within the cytoplasm, although no positive stainings were found in keratinized foci. In addition to the carcinoma cell nests, positivities for perlecan were observed in the stromal connective tissue surrounding the carcinoma cell nests, which was myxoid in HE-stained sections. In contrast to the core protein, HS chains were apparently not immunolocalized in carcinoma cell nests but only in vascular vessels in the stroma (Fig. 3G). mRNA signals for perlecan core protein were detected in carcinoma cells in an irregular fashion (Fig. 3H), while the messages were consistently observed in vascular endothelial cells, some fibroblasts, and inflammatory cells including macrophages (not shown).

In poorly differentiated squamous cell carcinomas (Fig. 3I), perlecan core protein was strongly immunolocalized in both carcinoma cells and stromal spaces with myxoid appearances (Fig. 3J). The staining of carcinoma cells was not only enhanced in the cell border but also seen diffusely in the cytoplasm. In addition, coalescent stainings for perlecan were frequently observed in the intercellular space. In narrow stromal spaces showing a myxoid appearance, both fibroblastic cells and interstitial connective tissues were strongly immunopositive for perlecan. HS chains were not so regularly immunolocalized but focally in the cytoplasm of carcinoma cells as well as in stromal fibroblastic or vascular endothelial cells and in extracellular matrices (Fig. 3K). mRNA signals for perlecan core protein

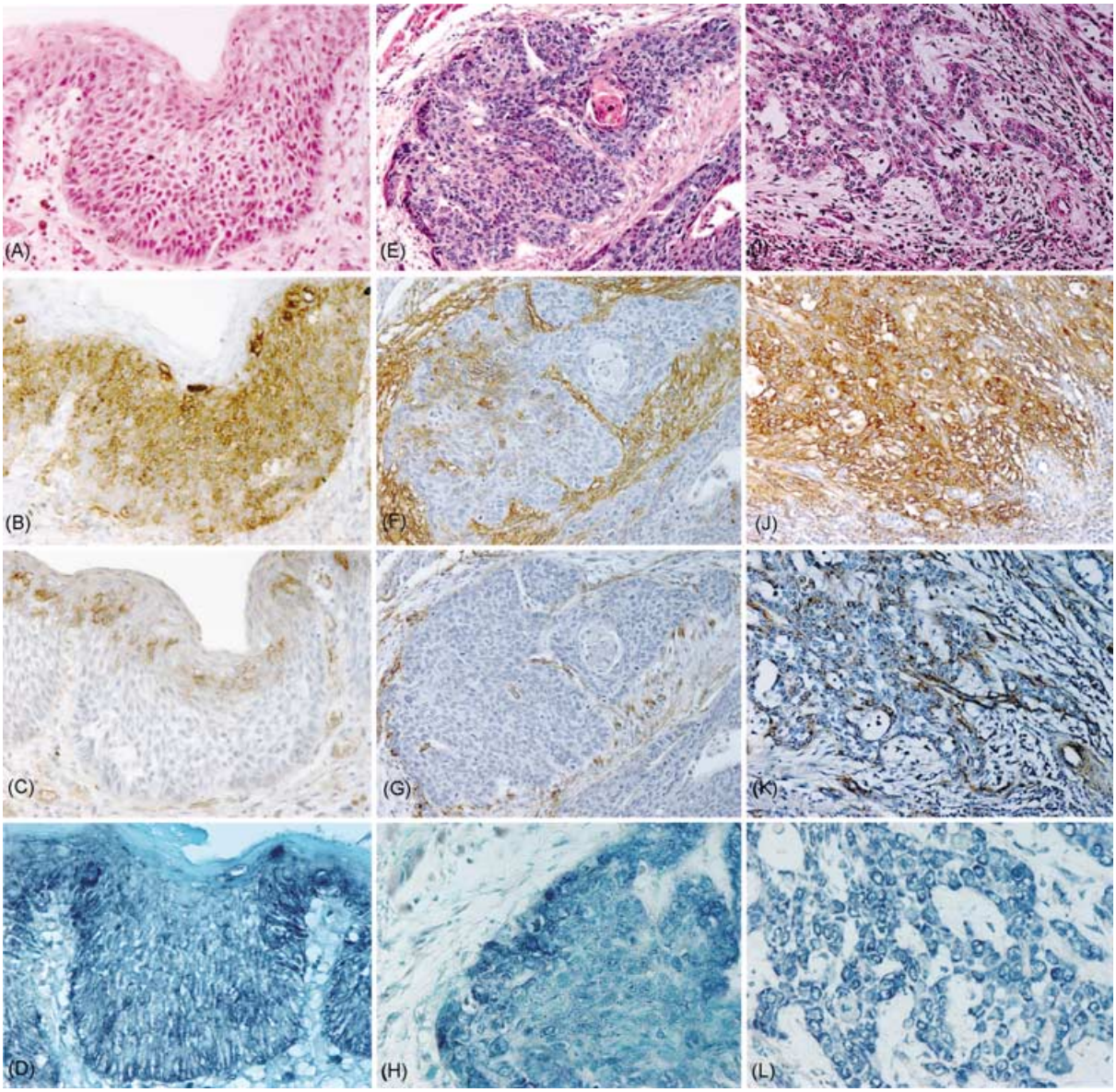

Figure 3 Epithelial dysplasia and squamous cell carcinoma of the oral mucosa. (A, E, I) HE stain, upper layer; (B, F, J) immunoperoxidase for perlecan core protein, second layer, hematoxylin counterstain; (C, G, K) immunoperoxidase for HS chains, third layer, hematoxylin counterstain; (D, H, L) *in situ* hybridization for perlecan core protein mRNA, lower layer, DIG-ALP, methyl green counterstain. (A–D) Severe dysplasia, left column; (E–H) well-differentiated squamous cell carcinoma, middle column; and (I–L) poorly differentiated squamous cell carcinoma, right column. A–D, H, L: ×250; E–G, I–K: ×125. Histology of severe dysplasia (A–D) was characterized by a massive proliferation of basaloid cells, which resulted in drop-shaped epithelial processes. Immunolocalization for perlecan core protein was intensively and widely expanded in the whole epithelial layer, not only in the cell border but also in the cytoplasm of the proliferating basaloid cells. In contrast, most of the immunolocalization for HS chains disappeared from the prickle cell layer, except for irregular patched deposits in the cytoplasm. mRNA signals for perlecan core protein were almost compatible. In well-differentiated squamous cell carcinomas (E–H), a larger focus represents squamous cell differentiation from basal cells in the periphery to keratinized cells in the center. Perlecan core protein was irregularly localized in the proliferating zone of carcinoma cells as shown in dysplastic epithelial cells but not in the keratinization center. In contrast, the HS chains were not recognized in the whole carcinomatous foci. In the stroma, positive stainings for HS chains as well as perlecan core protein were observed. mRNA signals for perlecan core protein were detected irregularly, but mainly in the periphery of the carcinoma cell nests. In poorly differentiated squamous cell carcinomas (I–L), perlecan core protein was strongly immunolocalized in both carcinoma cell strands and stromal spaces with myxoid appearances. HS chains were not so regularly immunolocalized but focally in the cytoplasm of carcinoma cells as well as in the stromal fibroblasts or vascular endothelial cells and in extracellular matrices. mRNA signals for perlecan core protein were localized strongly in the carcinoma cells as well as the stromal cells, which also showed immunopositivities for the core protein.

were localized strongly in the carcinoma cells as well as in the stromal cells, which showed immunopositivities for the core protein (Fig. 3L).

The immunopositivities for perlecan core protein and HS chains among the examined cases classified into normal, hyperplastic, dysplastic, and carcinomatous epithelia of the oral mucosa are summarized in Table 1. Figure 4 illustrates schematically comparative expression modes between perlecan core protein and HS chains in each state of the oral epithelium.

Table 1 Expression modes of perlecan core protein and heparan sulfate chains in oral epithelial lesions

		Ratio (%) of cases with immunopositives for							
		Perlecan core protein				*Heparan sulfate chains*			
				Prickle				*Prickle*	
Epithelial condition	*Sample no.*	*Basal*[a]	*Parabasal*[b]	*Lower*[c]	*Upper*[d]	*Basal*	*Parabasal*	*Lower*	*Upper*
Normal	10	10.0	90.0	20.0	0.0	80.0	80.0	70.0	10.0
Hyperplasia	10	30.0	100.0	100.0	50.0	70.0	80.0	90.0	80.0
Dysplasia									
Mild	22	81.8	100.0	100.0	59.1	59.1	90.9	95.5	31.8
Moderate	8	85.7	100.0	100.0	100.0	12.5	57.1	50.0	12.5
Severe	8	100.0	100.0	100.0	100.0	0.0	0.0	25.0	0.0
Squamous cell carcinoma									
Well-differentiated	18	22.2	27.3	13.6	13.6	33.3	33.3	0.0	0.0
Poorly differentiated	6	66.7	66.7	33.3	33.3	50.0	50.0	50.0	33.3

[a]Basal: basal cell layer; [b]parabasal: 2nd to 3rd layers; [c]lower prickle: 3rd to 5th layers; [d]upper prickle: 5th up to keratinized layers.

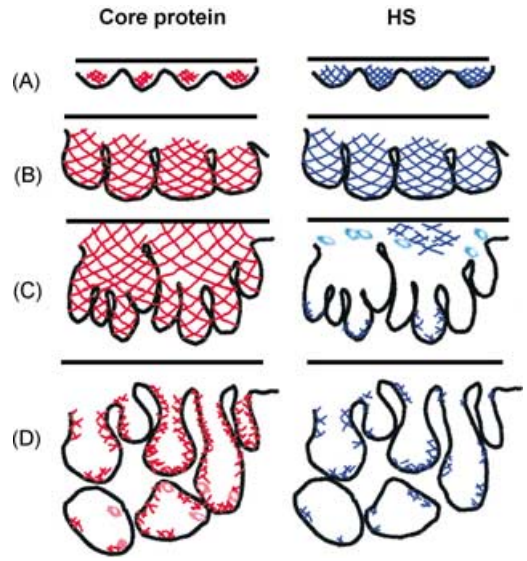

Figure 4 Scheme for comparative and differential expression modes between perlecan core protein and HS chains in the oral mucosal epithelium from normal to dysplastic and carcinomatous states. Perlecan core protein, left column and HS chains, right column. Upper layer (A), normal epithelium; second layer (B), mild epithelial dysplasia; third layer (C), moderate and severe epithelial dysplasia; and bottom layer (D), squamous cell carcinoma.

Discussion

In the present study, we were successful in demonstrating the differential expression modes of perlecan at its gene and protein levels in squamous epithelial cells of oral mucosal lesions. Its expression seemed to be on the cell border of parabasal cells in the normal or hyperplastic epithelium; hence, the parabasal cells seemed to be responsible for the production of perlecan in usual conditions. Furthermore, we could show that the perlecan expression was dynamically extended from its original position of the parabasal cell layer to both the upper and the lower layers with the increase in the severity of dysplastic changes of the epithelium. It is thus suggested that perlecan is synthesized by germ cells with parabasal cell-like appearances, which are the major constituent cells of epithelial dysplasia, and that perlecan is deposited in the intercellular space of dysplastic epithelial cells to serve their proliferation.

The extension of the perlecan expression from the parabasal cells to the basal and prickle cell layers with severity of dysplastic changes seemed to correspond generally with extensive growth of basaloid cells within the range of epithelial dysplasia. This result suggests that parabasal cells could be regarded as the most primitive cells in the oral mucosal epithelium, and that the basal cells, which do not express perlecan in a normal condition, should be interpreted as apparently differentiated cells that are aligned in a row on the basement membrane and interface with the lamina propriae connective tissue. The presence of HS chains in the basal cells of normal-to-mild dysplastic epithelia is thus thought to represent the presence of cell membrane HSPGs other than perlecan (15). Several lines of evidence by immunohistochemistry have shown that basal cells express specific cell membrane molecules, such as integrins (16), type II interleukin 1 receptor (17), epidermal growth factor (18), and fibroblast growth factor (FGF) (19). Differential lectin bindings in basal cells have also been demonstrated in oral epithelial dysplasias (20) and squamous epithelial linings of jaw cysts (21). These facts may indicate that basal cells should be distinguished from parabasal cells, and they are differentiated in terms of several apparent functions.

It is thus reasonable to consider that the proliferating cells in epithelial dysplasias are not differentiated as normal keratinocytes but maintain their primitive or parabasal cell-like characteristics. The immunolocalization for perlecan on the cell border is thought to possibly suggest its actual localization in the intercellular space of epithelial cells because perlecan is an extracellular matrix (ECM) molecule and is not thought to be assembled into the cell membrane (22).

As the intercellular deposits of perlecan became more apparent or irregular with the increase in dysplastic degrees, the biosynthesis of perlecan seemed to be accelerated in the process of malignant transformation of epithelial cells, which was still limited to the epithelial layer. As a result of enhanced intercellular deposition of perlecan, the intercellular space was apparently widened, and this may be interpreted histopathologically as ''loss of intercellular adherence,'' which is listed in the 13 histological changes that may contribute to a diagnosis of epithelial dysplasia in the WHO classification (11). The widening of intercellular spaces in epithelial dysplasia was pointed out by Saika et al. (23). Their ultrastructural study indicated that dysplastic epithelial cells exhibited abnormally wide spaces between the cytoplasmic projections, with desmosomes only formed on the apices of these projections. The increasing separation of epithelial cells was also quantitatively demonstrated in the process of experimental carcinogenesis of the hamster cheek pouch (24). The intercellular deposits of perlecan may result in a decrease in the number of desmosomes and gap junctions, which was demonstrated by Kocher et al. (25) in pre-cancerous and carcinomatous lesions of the skin as well as of the oral and cervix mucosas.

The intercellular accumulation of perlecan in dysplastic epithelia seems to disturb cellular communication between epithelial cells especially in the lateral direction, such as from basal cells to basal cells or from prickle cells to prickle cells. Such a reduction in communicating abilities of stratified squamous epithelial cells may contribute to their concordant differentiation towards keratinization. A most distinct example of the intercellular deposits of perlecan is the enamel pulp of the tooth germ (26). The stellate reticulum seems to have resulted from an excessive amount of proteoglycans including at least perlecan in the intercellular space. Proteoglycans tend to gather water molecules around them, which are thought to participate in the widening of the intercellular space, although the functional significance of this phenomenon is still controversial. Furthermore, the intraepithelial deposits of perlecan have already been demonstrated in the tumor cell nests of ameloblastomas, which mimic the enamel pulp appearance (11). As the gene expression of perlecan was shown to be enhanced in the invading front of ameloblastomas, its intercellular deposits may play an important role in cellular proliferation. Thus, the perlecan deposits in epithelial dysplasia suggest that dysplastic cells require perlecan, especially its core protein, for their proliferation. For this interpretation, a possible advantage of intercellular deposits of perlecan could be explained by one of its representative functions as a reservoir for several kinds of growth factors, such as basic FGF (FGF2) (27), FGF7 (28), or TGFβ (29). Trapped growth factors in the intercellular space because of the presence of perlecan may function in the proliferation of dysplastic epithelial cells. In fact, Wakulich et al. (30) have shown that the expression levels of FGF2 are in accordance with the severity of epithelial dysplasias, which were found in the vicinity of human head and neck squamous cell carcinomas. Hughes et al. (31) also reported increased levels of FGF2 mRNAs in oral dysplastic epithelial cells.

Another possible function of intraepithelial perlecan is suggested to be a space for migration of intraepithelial cells, such as lymphocytes and macrophages, including Langerhans cells, which are usually distributed in the epithelial layer of oral mucosa and are thought to patrol for immune stimulations from the oral cavity (32). However, their molecular mechanism of intraepithelial migration is poorly understood, although their widened intercellular space may make it easier for inflammatory cells to infiltrate into proliferating epithelial cell nests, which was suggested by White & Gohari (24). It is obvious that ECM molecules are required in cellular migration as substrates for adhesion and rolling, which has been suggested, however, only in the connective tissue space (33). Therefore, it is quite interesting to investigate further the role of intraepithelial perlecan as the substrate for immune-responsible cell mobility.

The irregular and ill-ordered expression of perlecan core protein and the simultaneous disappearance of its HS chains in squamous cell carcinomas partially correspond with the results from epithelial dysplasia. However, the large amount of intercellular deposits, which has been shown to be the hallmark of epithelial dysplasia, was not found within carcinoma cell nests. Instead, the stromal accumulation of perlecan was pronounced in squamous cell carcinomas. These contrastive results may indicate that perlecan is deposited in the intercellular space of dysplastic epithelial cells when their proliferation is retained within the epithelial layer where no stromal space is induced, but that carcinoma cells no longer need to secrete perlecan once they attain invasiveness and are able to induce perlecan-rich stromal matrices which are produced by stromal cells, alternatively.

The loss of intercellular HS chains in epithelial dysplasia contrasted significantly with the enhanced intercellular deposits of perlecan core protein in the epithelial layer. The results suggest three possibilities: (i) these deposited perlecan molecules were incomplete forms lacking HS chains by nature; (ii) HS chains of secreted perlecan molecules were degraded by HS lyases, such as heparanases; and (iii) HS chains synthesized by dysplastic cells were malformed and could not be recognized by the antibody F58-10E4, which only recognizes native forms of HS chains (34). Of these three speculations, the second one seems most likely because elevated levels of heparanase have already been demonstrated in dysplastic epithelia of the esophageal (15) and colon (35) mucosas. It is also well known that heparanase is one of the important degradative enzymes that plays an important role in invasion and metastasis of mouse lymphomas (36) and of human melanoma and rat breast carcinomas (37). The third speculation has been also proposed by van Kuppevelt et al. (38), who used phage display technology to generate anti-HS antibodies. Three different phage clones expressing anti-HS chain variable fragment antibodies were isolated; all three antibodies recognized different types of HS molecules as indicated by their different staining patterns and different reactivity towards heparitinase and chondroitinase treatments on rat kidney sections. In addition, HS chains have been shown to be structurally heterogenous because of their variabilities in the levels and distributions of sulfation and epimerization along the carbohydrate backbones (39). David et al. (34) also suggested a possibility of abnormal structures of HS chains expressed in hamster embryonal and adult kidney tissues because the 10E4 antibody did not work. The first

speculation for incomplete perlecan molecules cannot be ruled out either because perlecan core proteins without HS chains have been shown to be secreted by COS-7 cells when transfected by expression vectors containing only domains I, II, and III of the core protein cDNA (40). The same authors, using the same experimental system, also reported a phenomenon that smaller sized HS chains instead of the usual ones with a molecular mass of 70 kDa were attached to the core when domains I and II were over-expressed (41). In any case, it is obvious that the metabolisms of the core protein and HS chains are not synchronized in the process of intraepithelial malignant transformation of the epithelium.

In the present study, we confirmed for the first time that the intercellular space of epithelium does contain an ECM molecule such as perlecan that serves as stroma for individual epithelial cells. For the moment, perlecan is the only ECM molecule confirmed to exist in the epithelial layer, and it is highly suggested that other ECM molecules also exist and function within the epithelium, which should be examined in the next step.

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Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (B) and Exploratory Research from the Ministry or Education, Culture, Sports, Science and Technology and from Japan Society for the Promotion of Science.