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Original article

Differential immunohistochemical expression profiles of perlecan-binding growth factors in epithelial dysplasia, carcinoma in situ, and squamous cell carcinoma of the oral mucosa

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ABSTRACT

The intercellular deposit of perlecan, a basement-membrane type heparan sulfate proteoglycan, is considered to function as a growth factor reservoir and is enhanced in oral epithelial dysplasia and carcinoma in situ (CIS). However, it remains unknown which types of growth factors function in these perlecan-enriched epithelial conditions. The aim of this study was to determine immunohistochemically which growth factors were associated with perlecan in normal oral epithelia and in different epithelial lesions from dysplasia and CIS to squamous cell carcinoma (SCC). Eighty-one surgical tissue specimens of oral SCC containing different precancerous stages, along with ten of normal mucosa, were examined by immunohistochemistry for growth factors. In normal epithelia, perlecan and growth factors were not definitely expressed. In epithelial dysplasia, VEGF, SHH, KGF, Flt-1, and Flk-1were localized in the lower half of rete ridges (in concordance with perlecan, 33–100%), in which Ki-67 positive cells were densely packed. In CIS, perlecan and those growth factors/receptors were more strongly expressed in the cell proliferating zone (63–100%). In SCC, perlecan and KGF disappeared from carcinoma cells but emerged in the stromal space (65–100%), while VEGF, SHH, and VEGF receptors remained positive in SCC cells (0%). Immunofluorescence showed that the four growth factors were shown to be produced by three oral SCC cell lines and that their signals were partially overlapped with perlecan signals. The results indicate that perlecan and its binding growth factors are differentially expressed and function in specific manners before (dysplasia/CIS) and after (SCC) invasion of dysplasia/carcinoma cells.

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1. Introduction

It remains a challenge to make objective histopathological diagnoses of oral borderline malignancies from epithelial dysplasia and carcinoma in situ (CIS) to microinvasive squamous cell carcinomas (SCC) only on hematoxylin and eosin (HE) stained sections, as the conventional grading systems are too heavily dependent on the subjectivity of pathologists, which leads to considerable disagreement [1–3]. Recently, we have proposed that the characteristic two-phase appearance, which results from a sharp and contrastive layering of the upper keratinized cell layer and the lower half basaloid cells, is recognized in some particular histological types of epithelial dysplasia or CIS [4–12], and it could be an important histopathological hallmark of potentially malignant epithelial lesions even on HE sections. In the lower half of the two-phase epithelial dysplasia, composed of basaloid cells which are immunohistochemically positive for Ki-67 [5,6] as well as podoplanin [12,13], there are enriched intercellular deposits of extracellular matrix (ECM) molecules such as perlecan, a basement-membrane type heparan sulfate proteoglycan [14–18]. In addition, the basaloid cells in the lower half showed simultaneous loss of E-cadherin and nuclear translocation of β-catenin from the cell membrane, which indicates that those basaloid cells form a cell proliferating center in the lower half [6].

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To further confirm our hypothesis that the lower half of the two-phase epithelial dysplasia is a cell proliferation center and that its histopathological recognition is of considerable help for the objective differential diagnosis of oral borderline malignancies, we now consider it necessary to investigate the expression profiles of perlecan-binding growth factors in oral epithelial dysplasia and CIS comparatively in normal epithelia and SCC because perlecan has been known to be an important extracellular reservoir for several kinds of growth factors or cytokines [19] including vascular endothelial growth factor (VEGF) [20], sonic hedgehog (SHH) [21], or keratinocyte growth factor (KGF) [22,23]. VEGF, which acts on endothelial cells to promote angiogenesis, is also required for tumor cells to proliferate in a cell-autonomous and angiogenesis-independent manner [24]. It is known that the SHH signaling pathway regulates cell migration, proliferation, and apoptosis in several cancer cells from the skin, oral cavity, gastrointestinal tracts, urinary bladder, and lung [25]. KGF has been recognized as a mesenchymal cell-derived paracrine mediator of epithelial cell growth [26], but it is also known to stimulate various carcinoma cells from the biliary tract [27] and breast [28], though it has not been immunolocalized in SCC of the head and neck [29]. Thus, the expression modes of these molecules in oral SCC are somewhat controversial. Their pathophysiological functions also remain totally unknown during the oral precancerous stages, though perlecan has been suggested to function in epithelial dysplasia and CIS [6,9,14,16].

In this study, our aim was to determine comparative immunohistochemical profiles in oral mucosal epithelia ranging from normal to SCC among the following molecules: perlecan; Ki-67, a cell cycle marker; such perlecan-binding factors as KGF, SHH and VEGF; as well as VEGF receptors Flt-1 and Flk-1.

2. Materials and methods

2.1. Tissue materials

Eighty-one surgical specimens of SCC or CIS and 10 biopsy specimens of epulis of 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. Each specimen simultaneously contained histopathologically different lesions ranging from frankly invasive and well-differentiated SCC foci and foci of CIS, epithelial dysplasia, and epithelial hyperplasia to definitely normal epithelial parts. From these specimens, we selected 30 foci of normal and hyperplastic epithelia, 50 of moderate epithelial dysplasia with the characteristic two-phase appearance [4–6], 45 of CIS, and 30 of SCC, all of which were diagnosed on hematoxylin and eosin (HE) stained sections as well as on their immunohistochemically stained sections for keratin 13 (K13), a prickle cell marker; K19, a basal cell marker; Ki-67, a cell proliferation marker; and K17/K16, carcinoma cell markers, as we have described elsewhere [5–9]. The diagnostic criteria used in this study are described in a separate section. All the specimens were routinely fixed in 10% formalin and embedded in paraffin. Serial 3-μm sections were cut from paraffin blocks, and one set of the sections was stained with HE while the other sets were used for immunohistochemistry. The experimental protocol for analyzing surgical materials was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

2.2. Cells

SCC cell systems (ZK-1, ZK-2, and MK-1) were established from SCC arising in the tongue (ZK-1 and ZK-2) and gingiva (MK-1) [13]. SCC cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), which contained 10% fetal bovine serum (FBS) (Gibco), 50 μg/ml streptomycin, and 50 IU/ml penicillin (Gibco). They were incubated at 37 °C in a humidified 5% carbon dioxide/95% air atmosphere.

2.3. Antibodies

Polyclonal antibodies against the mouse basement membrane-type perlecan core protein were raised in rabbits as described elsewhere (diluted at 50 μg/ml) [14,16]. Mouse monoclonal antibodies against VEGF (clone C-1, IgG2a, 1:200), Flk-1 (A-3, IgG1, 1:300) and rabbit polyclonal antibodies against KGF (IgG, 1:50) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against Flt-1 (IgG, 1:2000) were obtained from Oncogene Research Products (La Jolla, CA, USA) and those against SHH (IgG, 1:100) were obtained from Abcam Inc. (Cambridge, UK). A mouse monoclonal antibody against human Ki-67 (MIB-1, IgG1, 1:50) was obtained from Dako (Glostrup, Denmark).

2.4. Immunohistochemistry

Paraffin sections were subjected to immunohistochemical stainings for perlecan core protein, VEGF, KGF, SHH, Flt-1, Flk-1, and Ki-67 by using the Envision+/HRP system (Dako). For VEGF, sections were treated with 0.15% trypsin (type II, Sigma Chemical Co., St Louis, MO, USA) in 10 mM Tris–HCl (pH 7.6) for 30 min at 37 °C. For SHH, Flt-1, Flk-1 and Ki-67, sections were autoclaved in citric acid buffer (pH 6.0) at 120 °C for 10 min. After that, the sections were rinsed in 0.01 M PBS containing 0.5% milk protein (Morinaga Milk Industry Co. Ltd., Tokyo, Japan) 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, sections were incubated with 5% milk protein in T-PBS for 1 h at room temperature to block non-specific protein-binding sites. They were then incubated with the primary antibodies overnight at 4 °C. After incubation, the sections were rinsed in T-PBS and incubated with the polymer-immune complexes (EnVision+peroxidase, rabbit/mouse, Dako) for 1 h at room temperature. After rinsing with T-PBS, they were treated with 0.02% 3,3′-diaminobenzimine (Dohjindo Laboratories, Kumamoto, Japan) 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 (Dako).

Following HE staining and immunohistochemistry examinations for K13, K19, K17, K16, and Ki-67, performed as described elsewhere [5–12], all of the focus samples were classified as (i) normal or hyperplastic epithelia, (ii) mild and moderate epithelial dysplasia, (iii) CIS, or (iv) SCC. We did not use the category of severe dysplasia because we considered that there was no objective distinction between so-called severe dysplasia and CIS [5].

2.5. Immunohistochemical evaluation

Foci of SCC, CIS, dysplasia, and normal epithelial parts were evaluated by extension and intensity of the immunohistochemical reactions for the three perlecan-binding molecules, VEGF, SHH, and KGF, and compared with those for perlecan. The staining was evaluated in four epithelial zones—basal, parabasal, lower prickle, and upper prickle layers as indicated in Figs. 1–3—for positive ratios. Each layer was considered positive (+) or not positive (−)

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