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Podoplanin expression profiles characteristic of odontogenic tumor-specific tissue architectures

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ABSTRACT

Podoplanin, a representative immunohistochemical marker for lymphatic endothelial cells, is also expressed in many other kinds of cancer cells, although its pathophysiological function is largely unknown. Our aim was to determine immunolocalization modes of podoplanin among odontogenic tumors to discuss possible roles of podoplanin in their characteristic tissue architecture formation. Immunohistochemical profiles of podoplanin were investigated in 40 surgical specimens from ameloblastoma (AM), adenomatoid odontogenic tumor (AOT), calcifying cystic odontogenic tumor (CCOT), and keratocystic odontogenic tumor (KCOT) in comparison with those of proliferating cell nuclear antigen (PCNA), integrin β1, fibronectin, and matrix metalloproteinase 9 (MMP-9). Podoplanin was localized in the basal cell layer or in the peripheral zone of AM foci. It was found in spindle-shaped tumor cells of AOT, in both the basal and polyhedral cells of CCOT, and in the basal and parabasal cells of KCOT linings. Podoplanin-positive (+) cells were located within areas of PCNA+ cells, and integrin β1 was localized in the cell membrane of podoplanin+ cells in the intercellular space where fibronectin and MMP-9 were deposited. In conclusion, podoplanin+ cells and areas in odontogenic tumors are in close associations with extracellular matrix signalings as well as cell proliferation.

Introduction

Podoplanin, a mucin-like transmembrane glycoprotein, was originally identified in glomerular podocytes [2]. Since its monoclonal antibody clone D2–40 became known to specifically recognize lymphatic endothelial cells, it has been regarded as an immunohistochemical marker for lymphatics [6,21]. In recent years, the expression of podoplanin has further been confirmed in various types of tumor cells, including squamous cell carcinomas (SCCs) [4,5,7,24,28,30,38,39] and embryonal tissues [16,29], suggesting its multifunctional, neoplastic- and embryonal-tissue specific properties. However, how precisely podoplanin functions in each pathophysiological event still remains unresolved.

In the field of oral pathology, podoplanin immunohistochemistry has been shown to be helpful in the histopathological grading of oral borderline malignancies, especially in making a distinction between carcinoma *in situ* (CIS) and epithelial dysplasia. Podoplanin-positive (+) cells are definitely distributed more widely in CIS and SCC foci, where podoplanin+ cells are overlapped with Ki-67+ cells, than in epithelial dysplasia foci in which podoplanin is scarcely expressed only in the basal layer [7]. Podoplanin has also been regarded as a biomarker useful in predicting the risk for cancer development [18] or poor prognosis of SCC [39] in the oral mucosa. In oral CIS and epithelial dysplasia, meanwhile, we have demonstrated that the Ki-67+ area, namely the cell proliferation center [1,20], is also characterized by the intercellular deposits of perlecan, an extracellular matrix (ECM) molecule, which results in widening of the intercellular space [15], indicating that perlecan functions in cell proliferation [19].

The characteristic stellate reticulum-like histology of ameloblastoma (AM) [11,12] and its original stellate reticulum in enamel organs of the tooth germ [13,14] have been shown to result from the intercellular deposits of perlecan as the intraepithelial stroma [1,11–15,19]. In addition, we have confirmed that ECM molecules, including perlecan, play an important role in tumor cell proliferation, as well as in the characteristic tissue architectures of such other odontogenic tumors as adenomatoid odontogenic tumor (AOT) [26] and keratocystic odontogenic tumor (KCOT) [34,35]. Accordingly, we wanted to determine how podoplanin is expressed in odontogenic tumors with such characteristic intercellular ECM-rich tissue architectures. Although overall expression modes of podoplanin have been reported in

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AM [10,40], KCOT [27,40], odontoma [9], and tooth germs [16,29], there has been no investigation for podoplanin in those tumors based on such a hypothesis as ours that intraepithelial ECM is related to cell proliferation.

The purpose of this study was to confirm differential immunohistochemical profiles of podoplanin among four odontogenic tumors – AM, AOT, KCOT, and calcifying cystic odontogenic tumor (CCOT) – to compare their expression profiles of podoplanin with those of proliferating cell nuclear antigen (PCNA), as well as ECM-related molecules, to elucidate the roles of podoplanin in the characteristic histological architectures of the four tumor types.

Materials and methods

Materials

Eighteen surgical specimens of AMs, 2 of AOTs, 5 of CCOTs, and 15 of KCOTs 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 13-year period from 1996 to 2008 after histopathological re-examinations of tissue sections. Of the 18 AM cases, 10 were categorized into follicular (FA) types, and 8 were plexiform (PA) types. The surgical samples were fixed in 10% formalin and decalcified with Planck Rychlo's solution [34]. They were then routinely processed and embedded in paraffin. Serial 4 μm paraffin sections were used for immunohistochemistry. The experimental protocol for analyzing surgical material was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

Antibodies

Mouse monoclonal antibodies against human podoplanin (clone D2–40, isotype IgG1) and PCNA (PC10, IgG2a) were obtained from Dako (Glostrup, Denmark). PCNA, instead of Ki-67, was adopted for identifying tumor cell proliferation activities because Ki-67 immunohistochemistry was not stable in decalcified tissue samples [35]. A mouse monoclonal antibody against human integrin β1 (4B7R, IgG1) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against human fibronectin were raised in rabbits as described elsewhere [3]. A mouse monoclonal antibody against human matrix metalloproteinase 9 (MMP-9) (56-2A4, IgG1) was obtained from Daiichi Fine Chemical Co., Ltd. (Toyama, Japan). For double immunofluorescence for podoplanin versus PCNA, Alexa Fluor 568 dye-conjugated goat antibodies against mouse IgG (for PDPN; Invitrogen, Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 488 dye-conjugated goat antibodies against mouse IgG (for PCNA; Invitrogen) served as secondary antibodies, respectively.

Immunohistochemistry

Immunohistochemical staining was performed by using the ChemMate Envision system (Dako) as described elsewhere [7,20]. For PCNA, sections were autoclaved in a citric acid buffer (pH 6.0) at 121 °C for 10 min [34]. For integrin β1, sections were pretreated with 0.2% trypsin (type II, Sigma–Aldrich Co, St. Louis, MO, USA) in 0.01 M Tris–HCl (pH 7.6) containing 0.1% $CaCl_2$ for 30 min at 37 °C [11]. For fibronectin, sections were pretreated with 3.0% hyaluronidase (bovine testicular origin, type I-S; Sigma–Aldrich) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 30 min at 37 °C [3]. After blocking endogenous peroxidase activities and non-specific protein binding sites, the sections were then incubated with the primary antibodies diluted at 1:50 (4B7R and 56-2A4), 1:100 (PC10), 1:200 (D2–40) and at 50 μg/ml (fibronectin). After further incubation with the secondary antibodies conjugated with peroxidase-labeled dextran polymers, reaction products were visualized with 3,3′-diaminobendimine. Finally, the sections were counterstained with hematoxylin. For control studies on antibodies, the primary antibodies were replaced with preimmune rabbit IgG or mouse IgG subclasses (Dako). For double-immunofluorescence studies for podoplanin and PCNA, the anti-PCNA antibodies were applied after indirect immunofluorescence signals for PDPN were stabilized by washing with 0.05 M glycine–HCl (pH 2.2) [7].

Results

Immunohistochemical profiles for podoplanin, PCNA, integrin β1, fibronectin, and MMP-9 were compared among the four types of odontogenic tumors as follows.

Ameloblastoma

In FA foci (Fig. 1a), podoplanin was localized distinctively on the cell surface of individual tall columnar cells located in the peripheral/basal cell zone (Fig. 1b). These aligned columnar cells were simultaneously and specifically positive for PCNA (Fig. 1c) and integrin β1 (Fig. 1d). Fibronectin was localized in the intercellular space of peripheral columnar cells, as well as in stellate reticulum-like cells in addition to the stromal space (Fig. 1e). MMP-9 was strongly expressed in the basal columnar cells and faintly in stellate reticulum-like cells (Fig. 1f). Thus, in FA, podoplanin was mainly expressed in the basal zone of tumor cell foci where proliferating cells and the turnover of ECM were observed.

In PA foci (Fig. 1g), podoplanin was also restricted to the peripheral/basal columnar cells and to some extent to the stellate reticulum-like cells (Fig. 1h). PCNA was positive in the basal columnar cells, as well as in the stellate reticulum-like cells, although the strongest staining intensities were obtained in the basal cells (Fig. 1i). Similar to the two previous molecules, integrin β1 appeared mainly in the basal cells with some spreading to the stellate reticulum-like cells (Fig. 1j). Fibronectin (Fig. 1k) and MMP-9 (Fig. 1l) were localized in the intercellular space of both the basal and stellate reticulum-like cells, while fibronectin was strongly deposited in the stromal space. In PA foci, podoplanin-positive (+) cells were basically overlapped by PCNA+ cells, as well as by ECM/ECM-related molecule+ cells except for stromal cells.

To confirm the co-localization of podoplanin and PCNA+ cells, double-immunofluorescence for the two molecules was performed. Both of the molecules were similarly distributed, though double-positive signals were more enhanced in the basal columnar cells (Fig. 2).

Adenomatoid odontogenic tumor

The histology of AOT was characterized by both whirl-like and pseudocystic (gland-like) foci, and the space between the two types of tumor cell foci was filled with spindle-shaped tumor cells (Fig. 3a). Podoplanin was characteristically localized in the cell surface of spindle-shaped tumor cells with some extension into the whirl-like foci, as well as to the lateral cell border of tall columnar cells forming pseudocysts (Fig. 3b). PCNA positivity was more predominant among the spindle-shaped cells, while it was not prominent in those forming the whirl-like structures; the tall columnar cells forming pseudocysts, especially those located in the basal (luminal) side, were positive for PCNA (Fig. 3c). Integrin β1 was exclusively localized in the spindle cells (Fig. 3d). Fibronectin (Fig. 3e) and MMP-9 (Fig. 3f) were similarly localized in most of the intercellular spaces of tumor cells.