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Original

Juxta-Epithelial Hyalinization Inhibits Tumor Growth and Invasion in Ameloblastoma

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Abstract:In histological examination of ameloblastoma, marked juxta-epithelial hyalinization of the connective tissue adjacent to the epithelium was observed in many cases. However, the physicochemical mechanism of these changes and its role in ameloblastoma is still not understood. To analyze the expression pattern of the different types of basement membrane related molecules, apoptosis-related factors and their possible role in juxta-epithelial hyalinization, six cases of ameloblastoma were examined. Immunohistochemical staining was done using cell surface type heparan sulfate (HS), 10E4, basement membrane type HS, JM403, Heparanase, Caspase-6, BCL-2 and CD34 antibodies. Hyalinized area in all sections, were strongly positive to 10E4, but negative to heparanase. There was no CD34 positive endothelial cells within the hyalinization area. Tumor cells adjacent to this area were positive to 10E4, heparanase and caspase-6. This result suggests that hyalinization have an effect on tumor growth and stop stromal-tumor cell interaction by the help of HSPGs, resulting in the inhibition of the function of heparanase and angiogenesis. Finally, tumor cells adjacent to the hyalinization undergoes program cell death.

Key words: Ameloblastoma, Hyalinization, Heparan sulfate proteoglycan (HSPG), Angiogenesis, Apoptosis.

Introduction

Ameloblastoma is the most frequently encountered, benign but locally invasive odontogenic tumor. Typical ameloblastoma begins insidiously as a central lesion of bone, which is slowly destructive, and ultimately expand the bone[1)]. Cellular invasion requires break down of the basement membrane and the surrounding extracellular matrix followed by the growth and proliferation of cells. The invasive ability of ameloblastoma is also thought to be related to the high proliferation rate of the tumor cells and/or the release of biologically active molecules produced by the tumor cells[2)].

Hyalinization refers to an alteration within the cells or in extracellular spaces, which gives a homogenous glassy eosinophilic appearance in Hematoxilin and Eosin (H&E) staining. This hyalinization may be extracellular or intracellular[3)]. This extracellular hyalinization may be the consequence of extracellular deposits by conglomeration of plasma proteins or basement membrane material, and amyloid or collagenous fibrous old scar may appears as hyalinized[3)]. In ameloblastoma, especially in the follicular type, marked juxta-epithelial hyalinization of the connective tissue adjacent to the epithelium are frequently observed[4)]. But the physicochemical mechanism is still not clear.

Heparan sulfate proteoglycans (HSPGs) constitute a group of ubiquitous extracellular matrix (ECM) macromolecules and are composed of a core protein and co-valently linked heparan sulfate (HS) sugar chain[5)]. HSPGs have certain important functional roles in ECM assembly and integrity. Their HS chains also sequester and interact with many biologically active molecules such as growth factors, cytokines, chemokines and cell adhesion-molecules[6,7)]. There are different types of HS chains, which exist within the cell surface as well as in the extracellular matrix. JM403 and 10E4 are the basement membrane type HS and cell surface type HS, respectively. These epitopes have a specific chemical structure with different localization pattern and their secretion pathway and function are also different[8,9)]. Heparanase is an endo-β-D-glucoronidase that specifically cleaves the HSPGs sugar chain[10,11)]. In ameloblastoma, heparanase shows increased expression at the mRNA level as well as the protein level, and it

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cleaves the HSPGs sugar chain to release attached molecules, resulting invasion and secondary morphological changes of ameloblastoma[12, 13]. There is no report about important basement membrane component, HSPGs and its degrading endoglycosidases that cleaves the HS chains, in extracellular hyalinization.

In multicellular organisms, the ability to regulate cell death is present in same extent to cell growth and differentiation. Programmed cell death mainly proceeds by apoptosis, a tightly controlled process to remove unwanted cells efficiently with characteristic cytoplasmic and nuclear condensation and DNA fragmentation[14]. Many pathway related to apoptosis induction has been reported. Caspase-6 is one of the important apoptosis molecules that cleavage the lamin-A in apoptotic signaling triggered by resveratrol (RSV)[15]. The family of BCL-2 related proteins constitutes one of the biologically most relevant classes of apoptosis-regulatory gene. Some study reported about the expression of these different kinds of apoptosis related molecules in ameloblastoma[16,17,18]. But the expression and subsequent role of these apoptosis family molecules in hyalinization area are not yet been reported.

Therefore, in this study, we attempted to analyze the expression pattern of the different types of basement membrane related molecules, apoptosis-related molecules and their role on adjacent tumor cells surrounded by the juxta-epithelial hyalinization.

Materials and methods

Tissue sample selection

Six cases embedded in paraffin were selected from the surgical pathology unit of the Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences of Okayama University, Japan. The samples were fixed in 10% neutral buffered formalin, decalcified with 10% formic acid if necessary and routinely processed and embedded in paraffin. Histological diagnosis of ameloblastoma was done by routine H&E staining slides, according to WHO histological typing odontogenic tumors (Pathology And Genetics of Head and Neck Tumors WHO Classification of Tumors, 2005).

Immunohistochemistry

Three-µm sections mounted on salinized slides were used for immunohistochemical staining. Briefly, sections were deparaffinized in series of xylene for 15 minutes and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was achieved by heat treatment using 10 mM citrate buffer solution pH 6.0 (for JM403, Heparanase, BCL-2 and Caspase-6 immunostaining), or by 0.1% trypsin treatment for 5 min (for 10E4 and CD34 immunostaining). After treatment with normal serum, the sections with primary antibodies were incubated 4ºC overnight. The tagging of primary antibody was achieved by subsequent application of anti-mouse IgG and avidin-biotin complexes (Mouse/Goat ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) or Envision peroxidase detecting reagent (Dako, Carpinteria, CA, USA). Visualization of immunohistochemical reaction was performed by developing the enzyme complex with DAB/ H_2O_2 solution (Histofine DAB substrate; Nichirei, Japan) and counterstained with Mayer's hematoxylin.

Results

Histology

Hyalinization was characterized by homogenous, glassy, eosinophilic appearance adjacent to ameloblastoma follicle (Figure 1a).

Immunohistochemical staining of juxta-epithelial hyalinization:

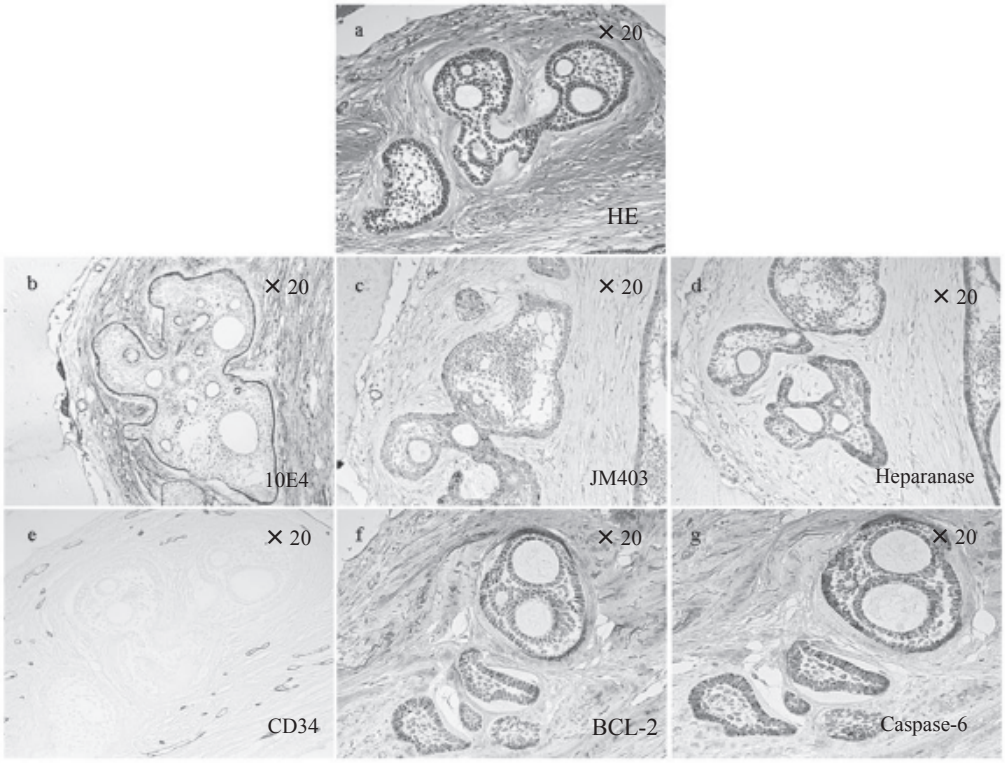
Cell surface type HS, 10E4, showed positive immunoreactivity in hyalinized area, as well as in surrounding connective tissue (Figure1b). Basement membrane type HS, JM403, was completely negative in hyalinization area, but surrounding connective tissue showed weak immunoreactivity by JM403 (Figure 1c). HS chain specific endoglucoronidase heparanase gave negative immunostaining in hyalinized area, but positive in surrounding connective tissue (Figure 1d). CD34, a specific endothelial cell markers, was completely negative in hyalinized area, whereas, blood vessels presents in the surrounding connective tissue was strongly positive by CD34 (Figure 1e).

Immunohistochemical staining of ameloblastoma follicle adjacent to the hyalinization

Cell surface type HS, 10E4, was observed clearly on the cell surface of peripheral columnar cells and also in basement

Table 1. List of the antibodies used in immunohistochemical study

Antibodies	Clonality	Supplier	Dilution
10E4	Mouse	Seikagaku Corp.Japan	1:500
JM403	Mouse	Seikagaku Corp.Japan	1:100
Heparanase	Mouse	Kindly provided by Dr. M.Nakajima	1:1500
BCL-2	Mouse	Dako, Denmark	1:50
Caspase-6	Mouse	Abcam Ltd, Cambridge, UK	1:50
CD34	Mouse	Nichirei, Tokyo, Japan	Ready to use



Immunohistochemical analysis of juxta-epithelial hyalinizzation

Figure 1a. Juxta-epithelial hyalinization in H & E stain (x100).
Figure 1b. Distribution patterns of cell surface type HS, 10E4, in hyalinized area and adjacent tumor cells (x100).
Figure 1c. Distribution patterns of basement membrane type HS, JM403, in hyalinized area and adjacent tumor cells (x100).
Figure 1d. Distribution patterns of heparanase in hyalinized area and adjacent tumor cells (x100).
Figure 1e. Distribution patterns of CD34 in hyalinized area and adjacent connective tissue (x100).
Figure 1f. Distribution pattern of BCL-2 in tumor cells adjacent to the hyalinization area (x100).
Figure 1g. Distribution pattern of caspase-6 in tumor cells adjacent to the hyalinization area (x100).

membrane, but almost negative in the cell surface of the central stellate-reticulum like cells (Figure 1b). JM403, basement membrane type HS, was intermittently positive in basement membrane of the tumor nest and also positive in intercellular spaces of some supra-basal cells as well as some central stellate reticulum-like cells (Figure 1c). Strong intensities of the heparanase observed in peripheral epithelial cells of tumor nest but weak immunoreactivity was observed in central stellate cells (Figure 1d). Apoptosis related molecules caspase-6 showed positive expression in peripheral cells of the tumor follicle surrounded by hyalinization (Figure 1g). BCL-2 expressed in both central and peripheral cells of the tumor follicle (Figure 1f).

Discussion

The importance of the tumor-stromal cells interaction is well established in tumorigenesis. Tumor cells live in a complex microenvironment includes ECM, diffusible growth factors, cytokines and verity of non-epithelial cells[19].

Ameloblastoma is a benign but locally invasive neoplasm consisting of proliferating odontogenic epithelium, which usually has a follicular or plexiform pattern, lying in a fibrous stroma[4]. In many ameloblastoma shows juxta-epithelial hyalinization.

HSPGs are distributed rampantly at cell surfaces and in ECM including basement membrane, which interact with various signaling molecules, cell adhesion molecules and other ECM and basement membrane molecules[20, 21]. Moreover, HS chains of these HSPGs sequester a multitude of bioactive proteins such as growth factors, morphogens, cytokines, chemokines, enzymes, and cell adhesion molecules and serve as the reservoir for these molecules[22]. It has been reported that, glypican-3, a cell-surface type HSPGs, inhibits the growth and progression of different types of cancers[23, 24]. In ameloblastoma, it has already been reported that HSPGs epitopes not only different according to their chemical structure but also their function and secretary pathway is completely different form each other[9]. Heparanase is an endoglycosidase,which releases angiogenic factors and accessory by fragments of HS from the tumor microenvironment and induces an angiogenic response in vivo. And facilitate tumor cell invasion, vascularization and survival, all critical events in tumor progression[25]. In this study, it was observed that at the juxta-

epithelial hyalinization, cell surface type HS, 10E4 was positive, whereas, hyalinized area was completely negative to basement membrane type HS, JM403 and heparanase. It suggests that even though tumor cell secretes heparanase, surrounding 10E4, existing in hyalines area inhibit the action of heparanase. As a result, tumor-stromal cell interaction was stop and heparanase secrete from the tumor cells cannot come in contact with the stromal cells. Those tumor nest couldn't invade into the surrounding tissue and also this results support that two chemically different type HSPGs sugar chain acts on tumor cells as different way [9].

Angiogenesis is a biological process by which new capillaries are formed from preexisting vessels. Tumor angiogenesis is critically important for the growth of solid tumors as tumors remain in dormant phase for a long time in the absence of the initiation of blood vessel formation[26]. Apoptosis is one of the main types of programmed cell death (PCD) and involves a series of biochemical events leading to a characteristic cell morphology and death[27]. Apoptosis depends on proteolytic enzymes called caspases, which cleaves specific intracellular proteins resulting cell death. Another major class of intracellular regulators of apoptosis is the BCL-2 family of proteins[27]. When an apoptosis stimulus activates, activity of BCL-2, an anti-apoptosis molecules was blocked, leading to apoptosis[27]. It was reported that that ameloblastoma has much more apoptosis-inhibiting protein than the apoptosis-modulating protein, which is the reason for the high survival activity of ameloblastoma, and this also one of the reasons for high recurrence rate[16]. Interestingly in this experiment it was observed that juxta-epithelial hyalinized area were completely devoid from blood vessels and tumor cells adjacent to the hyalinization area positive to both caspase-6 and BCL-2. However, though there was no blood vessel, the tumor cells might not get any nutrition. Resulting, apoptosis stimulus was activated and that may block anti-apoptotic property of BCL-2. And tumor cells adjacent to the hyalinization area may undergo apoptosis.

In summary, it was concluded that even though ameloblastoma is a locally aggressive type tumor, because of immune response against the tumor growth hyalinization area was formed at the stroma adjacent to the tumor nest as a secondary stromal change. This hyalinized area secretes large amount of cell surface type HS, 10E4, which may inhibit the tumor-stromal cell interaction. As a result, inhibition of tumor cell growth and invasion. Angiogenesis on that particular area was also stopped. Finally, the tumor cells adjacent to this hyalinization may undergo program cell death.

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