Heparanase and cyclooxygenase-2 gene and protein expressions during progression of oral epithelial dysplasia to carcinoma

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Abstract

Heparanase and cyclooxygenase-2 (COX-2) are 2 key enzymes that modulate diverse physiological processes during embryonic development and in adult life. Their deregulations have been implicated in the growth and progression of many cancer types. To date, comparatively little is known about the roles of these molecules during oral carcinogenesis. The aim of this study was to investigate the expression patterns of heparanase and COX-2 during progression of oral epithelial dysplasia (OED) to carcinoma. In situ hybridization and immunohistochemistry were performed on 5 cases of normal mucosa, 15 cases of OED, 5 cases of carcinoma in situ and/or microinvasive carcinoma, and 40 cases of oral squamous cell carcinoma (OSCC). Results demonstrated that heparanase and COX-2 messenger RNA and protein were absent in normal oral mucosa but were coexpressed in increasing intensity as OED progressed to OSCC. Concomitant heparanase- and COX-2–positive staining in the stromal cells suggests that OED/OSCC progression may be modulated by stromal–cancer cell interactions. Diffuse intense staining of poorly differentiated OSCC compared with staining localized to tumor nest periphery in well- and moderately differentiated OSCC suggests that heparanase and COX-2 overexpressions correlated with tumor grade. Strong expression of these enzymes in tumor cells at the advancing front suggests a role in local tumor spread. These results, taken together, suggest that heparanase and COX-2 might play complementary roles in the stepwise progression of OED to carcinoma.

Keywords: Heparanase; Cyclooxygenase-2; Oral epithelial dysplasia; Carcinoma in situ; Oral squamous cell carcinoma; Oral carcinogenesis

1. Introduction

Oral squamous cell carcinoma (OSCC) and its precursor lesions, that is, potentially malignant disorders, are an important health concern in many countries including East Asian nations like Japan \citep{1}. Because this disease is generally associated with a poor prognostic outcome, elucidation of its mechanisms of development and progression is a high priority, and identification of biomarkers involved in its malignant transformation from precancerous states is of paramount importance \citep{2}. Oral squamous cell carcinoma is one of the few cancer types where it is possible to obtain biopsies at all stages of cancer development and progression for purposes of investigating their underlying molecular mechanisms.
In recent years, 2 specific enzymes, heparanase and cyclooxygenase-2 (COX-2), produced by cancer cells have received considerable attention because of their critical roles during cancer growth and progression. The mammalian heparanase is an endo-β-D-glucuronidase that specifically cleaves the heparan sulfate (HS) side chain of HS proteoglycans (HSPGs), releasing HS-binding proteins, namely, growth factors, extracellular matrix molecules, cell-attachment proteins, enzymes and enzyme inhibitors essential for the regulation of physiological functions related to embryonic morphogenesis, wound healing, tissue repair, and inflammation [3]. Although heparanase activity is tightly regulated in the physiological state, in cancer, heparanase is over-expressed to facilitate local tumor spread and metastasis [4].

Cyclooxygenase, also known as prostaglandin endoperoxide synthase, is the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids [5]. It has 2 genes, COX-1 and COX-2, which have been cloned and known to share more than 60% identity at the amino acid level and have similar enzymatic activities. COX-1 is considered a housekeeping gene and expressed constitutively in most normal tissues. Prostanoids synthesized via the COX-1 pathway regulate many homeostatic body functions such as hemostasis, cytoprotection of gastric mucosa, vasodilatation in renal vessels, and platelet aggregation. In contrast, COX-2 is an inducible immediate-early gene, and its expression is induced by various stimuli such as growth factors and cytokines. The pathophysiological role of COX-2 includes modulation of inflammation, ovulation, and carcinogenesis [6].

Thus far, most studies have separately enumerated the clinicopathologic significance of these enzymes. Heparanase overexpression shows a positive correlation with increased tumor invasiveness, increased metastatic potential, and decreased survival rates in gastric [7], thyroid [8], pancreas [9], head and neck [10], and oral [11] cancers. Cyclooxygenase-2 overexpression is associated with a poor prognosis in colorectal [12], breast [13], oral [14,15], renal [16], adrenal gland [17], and liver cancers [18]. There are a few reports that examined heparanase expression during oral carcinogenesis [11,19-21] and COX-2 expression in human [22-26] and experimental oral carcinogenesis [27,28]. However, the coexpression patterns of these 2 molecules during the progression of oral epithelial dysplasia (OED) to carcinoma have not been elucidated. The aim of the present study was to examine the messenger RNA (mRNA) gene and protein expressions of heparanase and COX-2 in normal, dysplastic, and neoplastic oral mucosa in the hope that this would shed light on the relative roles of these 2 enzymes during oral carcinogenesis.

2. Materials and methods

2.1. Tissue samples

Archival formalin-fixed, paraffin-embedded tissue blocks of 5 normal oral mucosa, 15 OEDs (mild: n = 5; moderate: n = 5; severe: n = 5), 5 carcinomas in situ (CIS) contiguous with microinvasive carcinoma, and 40 OSCCs (well differentiated: n = 15; moderately differentiated: n = 15; poorly differentiated: n = 10) from the Department of Oral Pathology and Medicine, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan, were retrieved. Serial 4-μm-thick slices from each specimen were prepared for routine hematoxylin and eosin staining, immunohistochemistry, and in situ hybridization. All selected cases were histologically verified and diagnosed according to the criteria of the World Health Organization Classification of Tumors [29].

2.2. Monoclonal antibodies and kits

The primary antibodies used were antihuman mouse heparanase monoclonal antibody (Histofine; Nichirei Co Ltd, Tokyo, Japan), and monoclonal antibody 13H14 for COX-2 (IBL Co Ltd, Gunma, Japan). Histofine streptavidin-biotin peroxidase (SAB PO) kit and Histofine 3,3′-diaminobenzidine (DAB) substrate kit were also sourced from Nichirei Co Ltd.

2.3. Immunohistochemistry

Heparanase and COX-2 staining was performed using the streptavidin-biotin-peroxidase complex method. Briefly, 4-μm-thick slices mounted on silanized slides were deparaffinized, rehydrated, and immersed in 0.3% methanol containing 1% hydrogen peroxide for 30 minutes to block endogenous peroxidase and rinsed in 0.05 M tris-buffered saline (TBS) (5 minutes, 3 times) before immersing in blocking solution for 10 minutes at room temperature. Antigen retrieval in paraffin slices was achieved by heating 3 times in 10 mM citrate buffer solution (pH 6.0) in a microwave. After blocking of nonspecific reactivity with rabbit serum for 10 minutes at room temperature, the slices were incubated overnight at 4 °C with the antihuman heparanase (1:150) and anti–COX-2 (1:50) antibody. Identification of the distribution of the primary antibody was achieved by subsequent application of a biotinylated antiprimary antibody (Histofine SAB PO kit) and streptavidin-peroxidase (Histofine SAB PO kit). The antigenic sites were demonstrated by reacting sections with a mixture of DAB/H2O2 (Histofine DAB substrate kit). The nuclei were counterstained with Mayer hematoxylin. For negative control, the sections were reacted with normal rat serum or with the secondary antibody alone. All the control sections were negative. Positive staining controls were included for each antibody, and where present in the specimens, internal staining controls were also checked for appropriate reactions with each antibody.

2.4. Tissue and probe preparation for in situ hybridization

Digoxigenin-11-UTP-labeled antisense and sense complementary RNA probes for heparanase and COX-2 were kindly provided by Dr Motowo Nakajima (New Business
and Technology Transfer; Johnson & Johnson K.K., Tokyo, Japan). These probes were prepared using a digoxigenin-RNA Labeling and Detection Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions and were used for in situ hybridization.

2.5. In situ hybridization procedure

For in situ hybridization, 4 μm-thick slices were deparaffinized, pretreated with proteinase K (3 μg/mL in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 37 °C for 10 minutes, postfixed in 4% paraformaldehyde–0.1M phosphate buffer (pH 7.4) for 10 minutes, and immersed in 0.2 M HCl for 10 minutes. Then, acetylation was carried out by immersing the slices in freshly prepared 0.25% acetic anhydride–0.1 M triethanolamine–HCl (pH 8.0) for 10 minutes at room temperature. After washing, the slices were brought through an ascending series of alcohol, air dried, and used for in situ hybridization.

The hybridization solution containing 50% deionized formamide, 105 dextran sulfate, 1× Dehardt solution, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 250 μg/mL of Escherichia coli transfer RNA (proteinase treated) 10 mM dithiothreitol, and 0.1 to 2.0 mg/mL of digoxigenin-UTP-labeled RNA probe was used. The probe was placed on the slices and covered by paraffilm and was incubated. Hybridization was performed at 50°C for 16 hours in a moisture chamber. At the end of hybridization, the slices were incubated with 50% formamide in 2× saline-sodium citrate (SSC) for 30 minutes at 50°C to remove excess probe. The slides were incubated with 2× SSC and 0.2× SSC for 15 minutes twice at 50°C.

The washed slides were incubated with digoxigenin (DIG) buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 60 minutes at room temperature. After rinsing, the sections were blocked with 1.5% blocking reagent. The sections were then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes at room temperature. After washing, the slices were immersed in 0.25% acetic acid–0.1M phosphate buffer (pH 8.0) for 10 minutes, postfixed in 4% paraformaldehyde–0.1M trishydroxymethylaminomethane–HCl (pH 8.0) for 10 minutes at room temperature. After washing, the slices were immersed in freshly prepared 0.25% acetic anhydride–0.1 M triethanolamine–HCl (pH 8.0) for 10 minutes at room temperature. After rinsing, the slices were blocked with 1.5% blocking reagent. The sections were then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes at room temperature. After washing, the slices were immersed in freshly prepared 0.25% acetic anhydride–0.1 M triethanolamine–HCl (pH 8.0) for 10 minutes at room temperature. After rinsing, the slides were incubated with 2× SSC and 0.2× SSC for 15 minutes twice at 50°C.

The washed slides were incubated with digoxigenin (DIG) buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 60 minutes at room temperature. After rinsing, the sections were blocked with 1.5% blocking reagent. The sections were then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes and then treated with nitroblue tetrazolium salt and labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes at room temperature. After rinsing, the slices were blocked with 1.5% blocking reagent. The sections were then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes at room temperature. After rinsing, the slices were incubated with 2× SSC and 0.2× SSC for 15 minutes twice at 50°C.

The sections were nuclear stained with methyl green and 5-bromo-4-chloro-phosphate at 37°C for color development. The washed slides were incubated with digoxigenin (DIG) buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 60 minutes at room temperature. After rinsing, the sections were blocked with 1.5% blocking reagent. The sections were then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes and then treated with nitroblue tetrazolium salt and labeled antidigoxigenin antibody (1:800 dilution) for 30 minutes at room temperature. After rinsing, the slides were incubated with 2× SSC and 0.2× SSC for 15 minutes twice at 50°C.

In all 15 OEDs evaluated, heparanase and COX-2 mRNA signals were observed primarily in the basal, parabasal, and prickle cell layers, correlating with those areas expressing heparanase and COX-2 protein (Fig. 1B-F). The keratin layer was generally negative except for occasional small foci of expression (Fig. 1B-F). Moderate and severe OEDs including CIS/microinvasive carcinoma (Fig. 2A-D) were associated with stronger heparanase and COX-2 expressions than mild OED (Fig. 1B). In the underlying connective tissues, strong labeling was detected in the fibroblasts, inflammatory cells, and endothelial cells lining blood vessels and capillary sprouts.

3. Results

Immunohistochemical and in situ hybridization staining results for heparanase and COX-2 are summarized in Table 1 and illustrated in Figs. 1 to 4. Heparanase mRNA labeling was mainly nuclear, whereas its protein localization was predominantly membranous and cytoplasmic, being only occasionally nuclear. Cyclooxygenase-2 mRNA and protein localizations were nuclear and cytoplasmic, respectively. Heparanase and COX-2 expressions corroborated with each other.

### Table 1

<table>
<thead>
<tr>
<th>Oral epithelium</th>
<th>Heparanase mRNA</th>
<th>Protein</th>
<th>COX-2 mRNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Epithelial dysplasia (n = 15)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Mild (n = 5)</td>
<td>+</td>
<td>++</td>
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<td>Moderate (n = 5)</td>
<td>+</td>
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<td>++</td>
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<tr>
<td>Severe (n = 5)</td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
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<tr>
<td>CIS/Microinvasive carcinoma (n = 5)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 40)</td>
<td>+++</td>
<td>+++</td>
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(-), negative; (+), weakly positive in less than 25% cells; (+), positive in less than 50% cells; (++), moderately positive in less than 75% cells; (+++), strongly positive in more than 75% cells.
was observed (Fig. 3D-I). Tumor cells at the invasive front demonstrated strong labeling (Fig. 4A, B). The intervening stromal fibroblasts, endothelial cells, and mononuclear infiltrate were also stained positive. Dysplastic and normal mucosa at the tumor margin showed a gradual decrease in staining intensity for heparanase and COX-2.

4. Discussion

This is the first study to examine the expression profiles of heparanase and COX-2 mRNA and protein in oral epithelium during its progression from OED to carcinoma. The most significant finding was positive identification of these enzymes in increasing levels of intensity from OED and CIS to invasive carcinomatous epithelium. These findings suggest that overexpression of heparanase and COX-2 might be an early event (preinvasive stage) during oral carcinogenesis. Present observations correlated well with previous studies that separately investigated these 2 enzymes in human [11,14,15,24-26] and experimental oral cancers [19-21,30], in their precursor lesions [23,31], and during oral carcinogenesis [25,28].

In the present study, the preferential localizations of heparanase and COX-2 in the basal, parabasal and prickle cell layers of dysplastic oral epithelium were in agreement with earlier reports [23,25,28]. At each stage of progression, COX-2 expression pattern closely correlated with those of heparanase, suggesting that these 2 enzymes may play related roles during these transformation processes. Although morphologic information accrued from our immunohistochemical and in situ hybridization results alone could
not provide direct evidence as to the precise nature of their related roles, we speculated that the relationship between heparanase and COX-2 enzyme systems may be a significant one and that autocrine and paracrine mechanisms may probably be involved. Furthermore, because the mechanisms by which heparanase and COX-2 modulate tumor cell transformation and proliferation are not completely understood, evidence from studies by others [3,4] suggests that heparanase may act through 2 different pathways: (1) alteration of HSPGs on the epithelial cell surface resulting in the transformation of a normal cell into a tumor cell and/or (2) cleaving of HSPG-bound growth factors in the extracellular matrix such as transforming growth factor β, basic fibroblast growth factor, or vascular endothelial growth factor that promote or inhibit cell growth. On the other hand, COX-2 contributes to tumorigenesis via a 2-prong effect—direct effects on tumor cells by promoting mitogenesis and conversion of procarcinogen to carcinogens and indirect effects on nontumoral cells by nurturing tumoral blood vessels and modulating immunocompetent cells [32]. Similarity in expression patterns between these 2 enzymes has also been reported in colon carcinoma, where it has been suggested that heparanase modulates COX-2–mediated tumor angiogenesis [12]. Accordingly, during tumorigenesis, COX-2 induces angiogenic factors such as vascular endothelial growth factor and basic fibroblastic growth factor via the prostaglandin cascade [33], whereas heparanase releases these factors via degradation of HS, thereby promoting tumor angiogenesis and growth [3]. Although microvessel density scoring was not performed in this study, the observed concomitant strong heparanase and COX-2 labeling of endothelial cells lining young blood vessels and angiogenic sprouts in the stroma beneath dysplastic and carcinomatous epithelium indirectly supports this view [14,23,26].

In this study, heparanase and COX-2 were more strongly expressed in oral cancers than in dysplasias [14,23,24]. In the latter, staining intensity was positively correlated with the severity of dysplasia. These COX-2 findings correlated well with some oral cancer studies [23,24] but differed from the study by Shibata et al [26], who found that COX-2 labeling indices for dysplasias were higher than those for oral carcinomas. The mechanisms by which heparanase and COX-2 are overexpressed and up-regulated during oral carcinogenesis are still not clear. One report suggests that elevated levels of heparanase mRNA and protein in some human tumor cell lines and human head and neck tumors may be due to aberrant DNA demethylation [21] and that heparanase protein secretion is regulated by glycosylation [34]. In human prostate cancer, increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1
On the other hand, in breast, ovarian, and gastric cancers, up-regulation of COX-2 is speculated to be due to inactivation of intrinsic mechanisms such as inactivation of tumor suppressor genes like p53 and activation of proto-oncogenes like Ras and HER-2/neu and messenger RNA stability factor HuR.

In agreement with others, this study also demonstrated that heparanase and COX-2 expressions showed positive correlation with OSCC tumor grade. Poorly differentiated OSCCs have a strong, diffuse expression pattern, whereas in well-differentiated OSCC, heparanase and COX-2 were localized to the periphery of tumor nests. In contrast, present COX-2 findings differed from previous OSCC studies that found no relationship or an inverse correlation between COX-2 expression and tumor grade. It has been suggested that heparanase and COX-2 expressions may be lost with advanced cytodifferentiation of tumor cells probably as a result of alterations of their cell surface molecules. Our observation of absent to weak staining of keratinizing pearls supports this view.

Heparanase and COX-2 are well recognized as key enzymes in tumor invasion and metastasis. In this study, the demonstration of strong expressions of heparanase and COX-2 in tumor cells at the invasive front of OSCC further supports their role in local tumor spread. Absent to weak positivity in normal oral mucosa adjacent to OSCCs suggests that these enzymes may play a subsidiary role in the
paracrine regulatory mechanism of these enzymes during oral carcinogenesis [14].

In summary, this study examined heparanase and COX-2 mRNA gene and protein expression patterns in normal, dysplastic, and neoplastic oral mucosa in an attempt to clarify their roles during oral carcinogenesis. Our results revealed that both these enzymes were coexpressed, suggesting that they probably play complementary roles during oral carcinogenesis.

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References


