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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 [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 [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.

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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 overexpressed 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/H_2O_2 (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