***In vivo* localization of secretory type V phospholipase A₂ (sPLA₂-V) in human salivary glands under normal and pathological conditions**

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Abstract: Type V secretory phospholipase A₂ (sPLA₂-V) has been implicated in several pathophysiological events including inflammation, eicosanoid production, and antibacterial defense. However, its expression has been recognized in only a few organs such as the heart and lung. This study is the first to demonstrate preferential expression of sPLA₂-V in human salivary glands by immunohistochemistry and *in situ* hybridization. In normal tissues, sPLA₂-V was expressed in a constitutive manner in serous cells and scarcely in duct epithelia, but not in mucous cells. In contrast in sialoadenitis, sPLA₂-V expression was up-regulated in duct epithelia and some mucous cells, while the tumor cells of pleomorphic adenoma strongly expressed the enzyme. We concluded that sPLA₂-V in salivary glands may play a role for saliva secretions and anti-bacterial defense, in as well as assist in the progression of sialoadenitis and salivary gland tumor formation through prostaglandin production.

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Key words: COX-2, immunohistochemistry, *in situ* hybridization, salivary gland, serous acinar cells, sPLA₂-V

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Introduction

Phospholipase A₂ (PLA₂) enzymes catalyze the hydrolysis of membrane glycerophospholipids at the sn-2 position, liberating free fatty acids and lysophospholipids, and also play crucial roles in the regulation of arachidonic acid metabolism and phospholipid remodeling (1-2). Recent advances in molecular and cellular biology have led to the identification of more than 10 mammalian PLA₂ enzymes, which have been subdivided into three groups based upon their structures, enzymatic characteristics, subcellular distributions, and cellular functions (1-2). These include intracellular enzymes, such as Ca²⁺-dependent cytosolic PLA₂ (cPLA₂; type IV) and Ca²⁺-independent PLA₂ (iPLA₂; type VI), as well as secretory isoforms of PLA₂ (sPLA₂; types IB, IIA, IIC, IID, IIE, IIF, III, V, X, and VII).

Within the PLA₂ family, sPLA₂ enzymes are characterized by a common Ca²⁺-dependent catalytic mechanism, conserved disulfides, and a three-dimensional structure. The genes for six sPLA₂ types, IIA, IIC, IID, IIE, IIF, and V

(which compose the group II subfamily), are clustered at the same chromosomal locus (2). Of these, type V sPLA₂ (sPLA₂-V), similar to sPLA₂-IIA, is inducible by proinflammatory stimuli, and can substitute for sPLA₂-IIA in some cells (3-5). Further, the transcript of sPLA₂-V can be detected in the heart and lung, and in trace amounts in several other tissues (6-7). Functionally, sPLA₂-V is capable of augmenting eicosanoid production in several cells (3-5). Mammalian sPLA₂ enzymes are structurally similar to those in snake venom (types I and II), which are secreted from the salivary glands and possess hemolytic activity and other toxicity (7, 9). However, there are few reports concerning the expression and possible functions of sPLA₂ enzymes in human salivary glands.

In the present study, we examined sPLA₂-V localization in human salivary glands by immunohistochemistry and *in situ* hybridization. Based on our results, we also compared the localization of cyclooxygenase-2 (COX-2), downstream of PLA₂ enzyme in the prostaglandin-biosynthetic pathway, with the histopathological significance of sPLA₂-V.

Materials and Methods

Preparation of human salivary gland tissue sections

Human salivary gland tissues were obtained from the files of the Department of Pathology, Nihon University School of Dentistry. Specimens were collected from a total of 23 salivary glands, which included 5 parotid glands, 3 submandibular glands, and 5 buccal glands as normal salivary glands, 5 submandibular glands from cases of sialoadenitis and 5 parotid glands from cases of pleomorphic adenoma. Normal salivary glands were obtained from disease-free areas of tissue excised from patients who underwent surgical resections. All tissues were fixed with 10% neutral buffered formalin solution and embedded in paraffin.

Immunohistochemistry

Immunostaining was performed with rabbit anti-human sPLA₂-V and rabbit anti-human COX-2 (Santa Cruz Biotech, Santa Cruz, CA, USA). The human sPLA₂-V antibody was prepared by immunizing New Zealand white rabbits with recombinant sPLA₂-V (provided by Dr. Michael H. Gelb, University of Washington, Seattle, USA). The anti-sPLA₂-V antibody reacts specifically with human sPLA₂-V expressed in mammalian cells, but not with other human sPLA₂

enzymes (10). For immunostaining, thin sections were incubated for 20 min with 0.3% (v/v) H₂O₂ in methanol to block endogenous peroxidase and then rinsed in PBS, after which they were incubated in 10% (w/v) bovine serum albumin or non-immune rabbit serum in phosphate-buffered saline (PBS) for 15 min to reduce non-specific staining. Sections were incubated overnight with an appropriate dilution of the primary antibody ($\times 800$ for sPLA₂-V and $\times 200$ for COX-2). Antibodies were visualized using a Zymed HSP kit (Zymed Laboratories, San Francisco, CA, USA). Further processing was performed following the manufacturer's instructions. Briefly, sections were incubated with a biotinylated goat anti-rabbit secondary antibody for 30 min at room temperature. After washing 3 times with PBS, avidin-HRP was applied to the slide for 30 min, followed by washing 3 times with PBS again. The sections were developed in a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min. Finally, the sections were counterstained with Mayer's hematoxylin, washed in PBS, dehydrated in graded ethanol, and then mounted.

In situ hybridization

In situ hybridization methods were performed with the serial tissue sections using a Ventana HX system (Ventana Medical System, Inc., Tucson, AZ, USA) as previously

Fig. 1. Immunohistochemistry for type V secretory phospholipase A₂ (sPLA₂-V) and cyclooxygenase-2 (COX-2) in salivary glands under normal and inflammatory conditions. Immunoperoxidase stains for sPLA₂-V (a-b, d-f) and COX-2 (c) in normal parotid (a) and submandibular (b-c) glands as well as in sialadenitis of the submandibular gland (d-f), hematoxylin counterstain, bars = 100 μ m. sPLA₂-V immunopositive reactions were detected in serous acinar and duct epithelial cells with various levels of staining intensity in the parotid (a) and submandibular (b) glands, however, mucous acinar cells were not positive. COX-2 immunopositive reactions were detected in myoepithelial cells and the inter-lobular space (c). Increased staining intensity for sPLA₂-V was found in duct cells (d) as well as in mucous acinar cells (e) in sialoadenitis of the submandibular gland. Some sPLA₂-V-positive macrophages were identified in infiltrating inflammatory cells (f).

described (11). The sPLA₂-V specific probe was constructed by cloning a 500-bp NotI-XbaI fragment of human sPLA₂-V cDNA across the NotI-XbaI restriction sites of the Bluescript plasmid (Stratagene Corp., La Jolla, CA, USA). The sense and anti-sense cRNA probes for sPLA₂-V were synthesized from the T7 and SP6 promoters, on NotI- and XbaI-digested linearized plasmids, respectively. Sections were pre-treated and hybridized using a Ventana ribo map kit (Ventana) with an automated Ventana HX system. Detection of the hybrid was performed with a digoxigenin nucleic kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland), following the manufacturer's instructions. Signal detection with nitro blue tetrazolium salt was performed at 20°C for 30 hrs in the dark. Sections were then dehydrated through an ethanol series (30%, 50%, 70%, 90%, and 100% ethanol, 2 min each) and washed twice for 5 min in xylene before mounting with Malinol mounting medium (Muto Pure Chemicals Ltd. Tokyo, Japan).

Results

Immunohistochemistry

In normal salivary glands, sPLA₂-V expression was found in serous acinar cells from each salivary gland as well as in intercalated duct cells (Fig. 1a, parotid gland; Fig. 1b, submandibular gland), but not in mucous acinar cells. The distribution pattern of sPLA₂-V was homogenous in the cytoplasm of the serous cells, and there were no differences in its distribution pattern between major and minor salivary glands. In some minor salivary gland specimens, intra-lobular duct cells showed a much higher staining intensity than serous acinar cells. There were no morphological differences between sPLA₂-V-positive and -negative duct cells. The anti-COX-2 antibody showed positive staining in myoepithelium, whereas no staining was seen in serous or

mucous acinar cells from normal salivary glands (Fig. 1c).

The expression of sPLA₂-V in minor salivary glands with sialoadenitis was similar to that in serous acinar cells from normal salivary glands, while intercalated duct cells showed a relatively strong staining with uniform distribution in the cytoplasm (Fig. 1d). However, the staining intensity was elevated in specimens with sialoadenitis, with strong staining observed in some lobules with scattered distribution in minor salivary glands samples. In addition, mucous acinar cells contained detectable levels of sPLA₂-V (Fig. 1e), while macrophages that had migrated into the salivary glands showed a strong expression of sPLA₂-V (Fig. 1f). In addition, COX-2 was detected in some intercalated ducts, serous acinar cells, and mucous cells, though the staining intensity was varied. In cases of sialoadenitis, the infiltrated macrophages expressed sPLA₂-V at high levels (data not shown).

In pleomorphic adenoma, which was composed of a mixture of glandular epithelial and myoepithelial cells within the mesenchymal elements, sPLA₂-V expression was detected only sparsely among the parts with solid epithelial proliferation, and no expression was observed in myoepithelial cells, whereas marked expression was detected in solid epithelial sheets located at the periphery of the tumor (Fig. 2a), though none was seen in tumor cells arranged in a tubular pattern. Positive staining was also detected in the amorphous materials in the lumens of small ducts (Fig. 2c) and in some parts of the hyaline stroma. In addition, COX-2 staining was detected in the same portion of the solid epithelial sheets (Fig. 2b) that were also sPLA₂-V-positive. In contrast, amorphous materials in the lumens of small ducts showed negative staining for COX-2 (Fig. 2d).

All immunohistochemistry results are summarized in Table 1. sPLA₂-V was consistently expressed in serous acinar cells from normal salivary glands, whereas its

Fig. 2. Immunohistochemistry for sPLA₂-V and COX-2 in pleomorphic adenomas. Immunoperoxidase stains for sPLA₂-V (a, c) and COX-2 (b, d), hematoxylin counterstain, bars = 100 μ m. sPLA₂-V (a) and COX-2 (b) were over-expressed in tumor cells forming solid nests. sPLA₂-V was strongly positive in amorphous materials retained within duct-like lumina, while tumor cells forming duct-like structures were only weakly positive (c). COX-2 was not detected in the amorphous materials, while it was and few positive cells were found among ductal epithelia (d).

Table 1. Secretory type V phospholipase A₂ and cyclooxygenase-2 in human salivary glands

case No.	age & sex	salivary gland location	lesion type	staining intensity					
				PLA ₂ -V [†]			COX-2 [§]		
				DE ^{*1}	SC ^{*2}	MC ^{*3}	DE	SC	MC
1	65 F	parotid	DF ^{*4}	-	3+	-	3+	-	-
2	50 M	parotid	DF	1+	2+	-	3+	-	-
3	75 M	parotid	DF	-	1+	-	-	1+	-
4	80 F	parotid	DF	2+	3+	-	2+	-	-
5	63 M	parotid	DF	1+	1+	-	-	-	-
6	50 M	submandibular	DF	-	1+	-	-	1+	-
7	49 F	submandibular	DF	3+	3+	-	2+	-	-
8	52 F	submandibular	DF	2+	3+	-	1+	1+	-
9	61 M	buccal	DF	-	1+	-	3+	-	-
10	65 M	buccal	DF	-	2+	-	3+	-	-
11	73 M	buccal	DF	1+	3+	-	2+	1+	-
12	44 M	buccal	DF	-	3+	-	1+	-	-
13	53 F	buccal	DF	-	2+	-	3+	-	-
14	48 F	submandibular	SA ^{*5}	2+	1+	2+	3+	1+	-
15	52 F	submandibular	SA	3+	-	3+	3+	1+	-
16	38 M	submandibular	SA	1+	-	2+	3+	-	-
17	55 F	submandibular	SA	-	1+	1+	3+	-	-
18	32 M	submandibular	SA	1+	1+	2+	3+	-	-
				duct	solid nest		duct	solid nest	
19	57 M	parotid	pleo ^{*6}	1+	3+		1+	-	
20	38 M	parotid	pleo	2+	2+		1+	-	
21	29 M	parotid	pleo	1+	-		-	-	
22	41 F	parotid	pleo	-	2+		-	-	
23	36 F	parotid	pleo	1+	3+		1+	-	

[†]secretory type V phospholipase A₂[§]cyclooxygenase-2^{*1}duct epithelium^{*2}serous acinar cell^{*3}mucous acinar cell^{*4}disease free^{*5}sialoadenitis^{*6}pleomorphic adenoma

expression was undetectable in mucous acinar cells from all samples examined.

Assessment of the expression of sPLA₂-V mRNA by *in situ* hybridization in normal submandibular (Fig. 3) and parotid glands, as well as labial glands with sialadenitis, revealed that signals were detected in the cytoplasm of

serous acinar cells, but not in that of mucous cells. Positive signals were strongly detected within serous cells using the anti-sense probe (Fig. 3a), whereas they were minimal with the sense probe (Fig. 3b). Specimens without probes showed no signals (Fig. 3c). sPLA₂-V mRNA was also detected in intercalated ducts, though their signal intensity was much

Fig. 3. *In situ* hybridization for sPLA₂-V mRNA in the submandibular glands. Incubation with anti-sense (a) and sense (b) probes and without probes (c). Digoxigenin-immunoalkaline phosphatase stain, methyl green counterstain, bars = 100 μm. Strong signals were found in serous cells using anti-sense probes (a). No signals were seen using control sense probes (b) or without probes (c).

weaker than that observed in acinar cells.

Discussion

PLA₂ activity has been reported in acinar cells, duct epithelium, and apical plasma membranes fractions from salivary glands (12-15); however, *in vivo* expression patterns of particular PLA₂ enzymes in salivary glands have not been investigated. The present study is the first to demonstrate preferential sPLA₂-V expression in serous acinar cells and intercalated duct cells from human salivary glands by immunohistochemistry under normal and pathological conditions. In contrast, sPLA₂-V expression was undetectable in mucous acinar cells from normal salivary glands. The presence of an sPLA₂-V transcript in the parotid gland, as demonstrated by *in situ* hybridization, confirms our conclusion that salivary gland acinar cells and duct epithelium express sPLA₂-V.

The *in vivo* physiologic functions of sPLA₂-V in salivary glands have not been fully elucidated, though it has been reported that Ca²⁺-independent PLA₂ activity in the apical membrane of acinar cells may be involved in the exocytosis of secretory vesicles by facilitating the membrane fusion process [12-15]. Since the PLA₂ reaction products, free fatty acids and lysophospholipids, can facilitate membrane fusion (16), it is likely that sPLA₂ enzymes also participate in the regulation of exocytosis. Indeed, ectopic expression of sPLA₂-V or related sPLA₂-IIA was shown to enhance exocytosis in mast cells and neuroendocrine cells (17-18). In the present study, we found that sPLA₂-V was expressed in normal human parotid and submandibular glands under disease-free conditions. Thus, sPLA₂-V may also regulate saliva secretion from salivary glands, thereby enabling the release of various anti-microbial agents such as lysozyme, secretory IgA, and peroxides from serous acinar cells, which cover the mucosal surface as a part of the mucosal defense mechanism against invading pathogens. Furthermore, it is interesting to note that several sPLA₂s types display bactericidal activity by disrupting bacterial membranes (19-21), while sPLA₂-IIA, an enzyme originally purified from synovial fluid, can kill both Gram-positive and negative bacteria (19-21). It is abundant in human tears and seminal fluid, and is considered to exhibit anti-bacteria functions (22-23). Like sPLA₂-IIA, sPLA₂-V exerts bactericidal activity toward Gram-positive bacteria (20-21). Therefore, it is conceivable to speculate that sPLA₂-V, together with other saliva-derived anti-microbial agents in the salivary glands, participates in host defense mechanisms and pathological response.

Our immunohistochemistry results showed that the staining intensity of sPLA₂-V in normal salivary glands varies among individuals, though it was consistently expressed in serous acinar cells. Further, infiltrating macrophages exhibited strong sPLA₂-V staining in specimen with sialoadenitis, suggesting induction of the enzyme by inflammatory stimuli. In accordance with the findings, lipopolysaccharide (LPS) was shown to augment sPLA₂-V expression and attendant prostaglandin E₂ synthesis in a

mouse macrophage-like cell line (6). In the present study, sPLA₂-V expression was markedly elevated in duct epithelium, macrophages, and mucous cells from salivary glands under inflammatory conditions, which may have been stimulated by such cytokines as interleukin-1 β and tumor necrosis factor- α that had been released into the microenvironment (24). In addition, it is noteworthy that the distribution of COX-2 in normal salivary glands differed from that of sPLA₂-V in the present study. This result indicates that sPLA₂-V secreted from serous cells under inflammatory conditions may not play a role in the regulation of prostaglandin synthesis, but rather act as a bactericidal agent or a modifier of exocytosis, as discussed above.

An elevated expression of COX-2 has been demonstrated in many types of carcinomas including colorectal adenocarcinoma, mammary tumors, and mesothelioma (25-26). COX-2 involvement in the propagation of various cancers *in vivo* has also been reported in that studies used COX-2-selective inhibitors and COX-2 gene manipulated mice (25-28). Moreover, obsessive over-expression of COX-2 in several *in vitro* cell lines has been shown to facilitate cell growth and suppress apoptosis (29), while it was recently reported that an increase of COX-2 expression was recognized in various types of benign and malignant salivary gland tumors (30). Therefore, it is possible that COX-2 expressed in pleomorphic adenoma tissue exhibits a similar oncogenic function to that expressed in other types of tumors. In the present study, sPLA₂-V and COX-2 were co-localized in the solid part of tumor cells from pleomorphic adenoma specimen, which suggests that sPLA₂-V may be involved in prostaglandin synthesis and possibly tumor development in this situation. Collectively, those results indicated that the possible contribution of sPLA₂-V to COX-2-dependent prostaglandin production is confined to particular types of salivary gland tumors.

In summary, we demonstrated the *in vivo* localization of sPLA₂-V in salivary glands. The varied expression of sPLA₂-V in the tested tissues indicates that the enzyme may exhibit multiple functions under physiologic and pathologic conditions, including anti-bacterial defense, regulatory exocytosis, and prostanoid biosynthesis as well as have possible unidentified functions. Clarifying the functions of this enzyme under each circumstance is of considerable importance for understanding the physiology and pathology of human salivary glands.

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