The expression and localization of osteopontin in the mouse major salivary glands*

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Summary. The present study investigated the expression and distribution of osteopontin in the mouse major salivary glands. The level of osteopontin expression in the mouse submandibular gland was higher (12.7-fold) than that in parotid and sublingual glands at the mRNA level. By Western blot analysis, intense positive bands were seen at the predicted molecular mass (about 55kDa) in all the major salivary glands, while an approximately 30kDa band of osteopontin was detected only in the submandibular gland. Indirect immunofluorescent and immuno-electron microscopy analyses demonstrated the localization of osteopontin in the luminal (apical) membranes of acinar cells in all the salivary glands. Osteopontin was also localized at the lumen of acini in the submandibular gland. These results suggest that the expression of osteopontin in the submandibular gland is different from that in the parotid and sublingual glands and that osteopontin may be degraded in the mouse submandibular gland.

Introduction

Osteopontin is a sialic acid-rich glycoprophosphoprotein (Fedarko *et al.*, 2004; Ogbureke and Fisher, 2004). This non-collagenous protein with an integrin-binding RGD sequence plays a significant role in the development and remodeling of bone tissue (Denhardt and Noda, 1998; Terai *et al.*, 1999). Osteopontin has generally been considered to be limited to bones and teeth in normal adults. Several studies, however, have shown that osteopontin is expressed not only in hard tissues but also in other soft tissues, including the kidney and the mammary gland (Denhardt and Guo, 1993; Brown *et al.*, 1994; Rittling and Novick, 1997; Fisher *et al.*, 2002). A high expression of osteopontin in the kidney particularly suggests the role of this protein in the inhibition of calcification in the urinary tract (Hudkins *et al.*, 1999; Hampel *et al.*, 2003). In addition, osteopontin is considered to have several functions such as cell migration, cell survival, regulation of immune cell functions, and the control of tumor phenotype (Weber, 2001). The spontaneous increment of osteopontin is also found in various diseases, including immunological disorders, neoplastic transformation, the progression of metastasis, and wound healing (Weber, 2001). In terms of expression and localization, it remains to be clarified whether or not osteopontin is expressed in salivary glands (Brown *et al.*, 1992; Kusafuka *et al.*, 1999; Ohtsuka *et al.*, 2000; Muramatsu *et al.*, 2002; Ogbureke and Fisher, 2004). Ogbureke and Fisher (2004) showed its localization in the cytoplasm of both acinar and ductal cells of mouse salivary glands. It was previously demonstrated that osteopontin mRNA is highly expressed in the mouse salivary glands (Ohtsuka *et al.*, 2000). However, no comparison of its expression between the major salivary

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glands has been carried out as yet. Muramatsu *et al.* (2002) demonstrated the localization of osteopontin at luminal sites in acinar cells in mouse parotid and submandibular glands. Nevertheless, both the detailed expression and localization of osteopontin remain to be elucidated in the mouse major salivary glands, and no report has been published on the mRNA expression and ultrastructural localization of osteopontin in the different salivary glands. Furthermore, the function of osteopontin in the major salivary glands remains unclear.

The purpose of the present study was therefore to elucidate the expression and localization of osteopontin in the mouse major salivary glands at both the RNA and protein levels and to discuss the function of osteopontin in the salivary glands.

Materials and Methods

All experiments were carried out according to the Guidelines for the Treatment of Animals established by Tokyo Dental College. Six-week-old male BALB/c mice were used in the study. The mice were anesthetized intraperitoneally with 100 mg/kg of 2.5 % sodium thiopental before the experiments.

RNA isolation and quantitative real time RT-PCR (QRT-PCR)

After extraction of the salivary glands (n=7), total RNA was extracted using the acid guanidinium-thiocyanate-phenol-chloroform (AGPC) method with TRIzol® (Invitrogen, Grand Island, NY, USA), and cDNA synthesis was performed as described in our previous reports (Ihara *et al.*, 2000). QRT-PCR was carried out using SYBR Green and AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA, USA)

according to our previous reports (Ohtsuka *et al.*, 2000; Muramatsu *et al.*, 2005). Primers for mouse osteopontin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used; the sequences of the primers are shown in Table 1. Amplification was then performed in duplicate using the primer sets in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA) with denaturation for 10 min at 95°C, followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing or extension at 60°C for 1 min. The threshold cycle (Ct) value for each reaction was calculated and relative quantitation ($\Delta\Delta C_t$ method) was carried out as described in our previous reports (Ohtsuka *et al.*, 2000; Muramatsu *et al.*, 2005).

Western blot analysis

The different major salivary glands (n=8) — parotid, submandibular, and submandibular — were homogenized and lysed in RIPA buffer (1 % Nonidet P-40, 150 mM NaCl, and 50 mM Tris [pH=7.4]) plus inhibitors (2 µg/ml aprotinin, 1 µM MG132, 200 µM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 60 µM leupeptin). Each lysate was boiled with a sample buffer and run on 7.5% SDS-PAGE. For Western blot, the gels were transferred onto PVDF membranes and blocked with Tris buffer saline with 0.1% Tween 20 (TBS-T) containing 3% milk. The membranes were incubated with an anti-osteopontin polyclonal antibody (dilution 1:250, IBL, Fujioka) and anti-actin (dilution 1:5000, Sigma, St. Louis, MI, USA) at 4°C overnight. After washing with TBS-T for 15 min three times, the membranes were incubated with HRP-conjugated anti-rabbit IgG (1:1000; Amersham Biosciences, Piscataway, NJ, USA) at room temperature (RT) for 1 h. Signal detection was carried out with the Amersham Biosciences ECL System (Amersham Biosciences).

Table 1. Primer sequences for the detection of specific mouse osteopontin and GAPDH

	Primer sequences	Product size
Osteopontin	F: 5' -TAGTGCCACAGATGAGGACCT - 3'	101 bp
	R: 5' -CAGAGGGCATGCTCAGAAGCT - 3'	
GAPDH	F: 5' - TGCCCAGAACATCATCCCTG - 3'	131 bp
	R: 5' - TCAGATCCACGACGGACGGACACA - 3'	

Immunofluorescence

Indirect immunofluorescence was carried out on the unfixed cryosections according to our previous method (Muramatsu *et al.*, 1996; Lee *et al.*, 1998; Ihara *et al.*, 2000). The major salivary glands (n=10) were embedded in O.C.T. compound (Tissue Tek; Miles, Elkhart, IN, USA) and quickly frozen with cold n-hexane (-80°C). They were sectioned to an approximately 6- μm thickness at -20°C , and non-specific binding was blocked with 10% normal goat serum for 30 min at RT. The sections were then incubated with the anti-osteopontin antibody (dilution 1:250) at 4°C overnight. After incubation with the primary antibody, they were washed in PBS for 5 min three times, and then incubated again for 2 h at RT with an

Alxa488-conjugated anti-rabbit antibody (dilution 1:200; Molecular Probes, Eugene, OR, USA). The specimens were observed under a fluorescent microscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany). As a negative control, immunofluorescence was also carried out without the primary antibody.

Immuno-electron microscopy

Perfusion-fixation with the 4% paraformaldehyde-0.1M phosphate buffer (PB) was performed transcardially, and the major salivary glands (n=10) were removed. The samples were cut into small pieces and post-fixed in the same fixative for 5 h. For post-embedding immuno-electron microscopic analysis, small pieces of the salivary

Fig. 1. Expression of osteopontin among the mouse three major salivary glands. Based on value of the parotid gland (value of 1), relative expression levels of osteopontin mRNA in the submandibular and the sublingual glands have been calculated. Osteopontin in the submandibular gland is expressed at a higher level (12.7-fold) than that in the parotid gland, and there is a statistical significance ($p<0.01$). Osteopontin in the sublingual gland is 1.1-fold, and thus approximately equal to that in the parotid gland.

Fig. 2. Western blot analysis of osteopontin in the mouse major salivary glands. Western blot analysis shows a band indicating osteopontin at the predicted molecular mass (about 55kDa) in the parotid, submandibular, and sublingual glands. Cross-reacted bands around 30 kDa also are detected, but only in the submandibular gland.

Fig. 3. Immunofluorescence of osteopontin in the parotid gland. Immunoreactivity of osteopontin is localized at acini, but not at ducts. D: duct. Bar = 200 μ m

Fig. 4. Immunofluorescence of osteopontin in the parotid gland. Osteopontin reaction is found at the luminal membrane sites in acinar cells. The reactive shapes are oval or cord-like. Bar = 30 μ m

Fig. 5. Immunofluorescence of osteopontin in the mouse sublingual gland. The reactive pattern in this gland is similar to that in the parotid gland. The shapes of the reaction site are oval or tube-like. Bar = 100 μ m

Fig. 6. Immunofluorescence of osteopontin in the mouse submandibular gland. Osteopontin expression in acinar cells of this gland is rather weaker than that in both the parotid and sublingual glands. Immunoreaction is also seen not only at the luminal membrane but also in the lumen (arrowheads). Bar = 100 μ m

gland were dehydrated and embedded in EPON 812 (TAAB, Reading, UK). Selected areas were trimmed under a light microscope and ultrathin sections (80 nm) were mounted on mesh grids. The sections were rinsed in PBS at 4°C overnight and then blocked with 10 % normal goat serum. The primary antibody for osteopontin was diluted to 1:250 and the sections were incubated at 4°C

overnight. They were washed in PBS for 6 h at RT and incubated with 5-nm colloidal gold conjugated anti-rabbit IgG (BBInternational, Llanishen, Cardiff, UK; dilution 1:20) at 4°C overnight. The sections were then examined under a transmission electron microscope (Hitachi H-7100, Tokyo) after staining with uranyl acetate and lead citrate.

Fig. 7. Electron micrograph of a part of the acinar cell in the mouse parotid gland immunostained for osteopontin. Gold labeling is associated with protuberant microvilli of the luminal membrane in the acinar cell. L: lumen, SG: secretory granule. Bar = 0.1 μ m

Fig. 8. Electron micrograph of an acinar cell in the mouse submandibular gland immunostained for osteopontin. Gold labeling is seen at luminal membrane in the acinar cell, and labeled contents are diffusely spread in the lumen. L: lumen, SG: secretory granule. Bar = 0.1 μ m

Statistical analysis

The expression of osteopontin at the RNA level was statistically compared between each gland using Tukey's HSD-test ($p < 0.01$).

Results

QRT-PCR

In QRT-PCR studies, each value was expressed as the mean of a duplicate to determine the expression level of osteopontin in the mouse major salivary glands. The referent was the parotid gland, assigned a value of 1, and the relative expression levels of osteopontin mRNA in the submandibular and sublingual glands were calculated, based on the value of the parotid gland. Osteopontin in the mouse submandibular gland was expressed at a higher level (12.7-fold) than that in the parotid gland, although in the sublingual gland, it was 1.1-fold — and thus approximately equal — to that in the parotid gland (Fig. 1). There was a statistical significance between the submandibular gland and parotid gland, and between the submandibular gland and sublingual gland (Fig. 1).

Western blot

In Western blot analysis, a band indicating osteopontin at the predicted molecular weight (about 55 kDa) was detected using the total lysate from the mouse parotid, submandibular, and sublingual glands (Fig. 2). This 55 kDa-band was faintly observed in the mouse submandibular gland, while the level in the sublingual gland was equivalent to that in the parotid gland. On the other hand, cross-reacted bands were seen at around approximately 30 kDa; they were intensively detected in the submandibular gland (Fig. 2) although they were not detected in either the parotid or sublingual glands. The expression level of the 30 kDa-band was weaker than that of the 55 kDa-band in the submandibular gland.

Immunofluorescence

Immunofluorescent analysis revealed the osteopontin reactivity in all the salivary glands, with localization at luminal (apical) sites in the acinar cells in the parotid gland (Fig. 3). The reactive shape was round, oval, or cord-like (Fig. 4). The pattern in the sublingual gland was similar to that in the sublingual gland (Fig. 5). However, osteopontin expression in the acinar cells of the mouse submandibular gland was rather weaker than that in both the parotid and sublingual glands (Fig.

6). Immunoreaction was also seen in the lumen of the submandibular gland (Fig. 6). No expression was found in the sections of the negative controls or in the ductal and myoepithelial cells around acini in the salivary glands.

Immuno-electron microscopy

Gold particles were found on or near the surface of the luminal membrane of acinar cells of the parotid gland, indicating the presence of osteopontin. This gold labeling was often associated with protuberant microvilli on the luminal membrane of the acinar cells (Fig. 7). The pattern of the labeling in the sublingual gland was similar to that in the parotid gland (not shown). The gold labeling was also seen on microvilli of the luminal membrane in the submandibular gland as well as that in the parotid gland. However, the gold particles were very sparse on the luminal membrane, and the labeled contents were diffusely spread into the lumen of the submandibular gland (Fig. 8).

Discussion

The expression of osteopontin in salivary glands has remained a subject to be clarified (Brown *et al.*, 1992; Kusafuka *et al.*, 1999; Ohtsuka *et al.*, 2000; Muramatsu *et al.*, 2002; Ogbureke and Fisher, 2004). Kusafuka *et al.* (1999) demonstrated that osteopontin was not expressed in human salivary glands, while Brown *et al.* (1992) reported that osteopontin was localized in their ductal and mucinous epithelia. On the other hand, Ogbureke and Fisher (2004) showed that osteopontin was distinct in both acinar and ductal cells in mouse salivary glands, and indicated that osteopontin was localized in the acinar cytoplasm of these glands as well as in the basal portion of ductal cells in human salivary glands (Ogbureke and Fisher, 2004). The present study, however, clearly demonstrated the presence of osteopontin at luminal sites in acinar cells but not in ductal cells in mouse salivary glands. This finding may also suggest that the localization of osteopontin is different between human and mouse.

Our Western blot analysis clearly revealed a 30 kDa band in the submandibular gland, while 55 kDa bands were distinct in all salivary glands. This was unexpected because osteopontin was highly expressed in the mouse submandibular gland at the mRNA level. It was surmised that osteopontin might be degraded at the protein level by proteinases in the submandibular gland. Agnihotri *et al.* (2001) reported that osteopontin was cleaved by matrix metalloproteinase (MMP)-3 or -7, resulting in the formation of a C-terminal fragment with 30 kDa.

Muramatsu *et al.* (2002) immunohistochemically demonstrated the presence of the active forms of MMP-3 and MMP-7 in the mouse submandibular gland, and Ogbureke and Fisher (2004) indicated that MMP-3 was localized in the acinar cells of the submandibular gland. These results suggest that either MMP-3 or -7 is involved in the degradation of osteopontin in the mouse submandibular gland.

A few studies have reported the ultrastructural localization of osteopontin in different tissues (Qu *et al.*, 1994, 1997; Madsen *et al.*, 1997; Hudkins *et al.*, 1999; Hampel *et al.*, 2003). These earlier reports using immuno-electron microscopy showed that osteopontin was strongly localized in the luminal membrane of human cells in the stomach (Qu *et al.*, 1997), gallbladder (Qu *et al.*, 1994), intestine (Qu and Dvorak, 1997), and kidney (Madsen *et al.*, 1997). Furthermore, Qu *et al.* demonstrated the localization of osteopontin in secretory granules of human mucous cells, and the microvilli of the membranes in human intestinal, gallbladder, and gastric epithelial cells (Qu *et al.*, 1994, 1997; Qu and Dvorak, 1997). Our immuno-electron microscopic study showed that osteopontin was localized on or near the luminal membrane of acinar cells. This luminal localization suggests the binding of osteopontin to integrin receptors on luminal membranes (Brown *et al.*, 1992) as well as the localization of integrin alpha at luminal sites in acinar cells in salivary glands (Delporte *et al.*, 1997). The saliva secretion process is divided into three steps: docking, priming, and fusion of the luminal membrane and secretory granules. The last fusion step in most regulated exocytosis is triggered by cytosolic free calcium (Fujita-Yoshigaki, 1998, 2000). Osteopontin has binding sites for both integrin alpha(v)beta3 and calcium (Denhardt and Noda, 1998). Taken together, these findings imply that osteopontin contributes to exocytosis, particularly in fusion between the luminal membrane and secretory granules.

The present study was unable to determine why expression of osteopontin in the mouse submandibular gland differs from that of both the parotid and the sublingual glands. Based on the results of the present study, 30-kDa osteopontin might be drained into the saliva of submandibular gland, while 55-kDa osteopontin is preserved in the luminal membranes of acinar cells.

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