EFTEM cytochemistry and sexual dimorphism of secretory granules in male and female hamster submandibular glands

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Key Words: hamster, submandibular gland, male, female, peroxidase, EFTEM

Summary: After glutaraldehyde fixation followed by osmium tetroxide postfixing, the secretory granules of acinar cells in male hamster submandibular glands (SGs) exhibit a characteristic bipartite substructure, with an electron-lucid rim and a more electron-dense central core. In female hamsters, the reverse is seen, with the larger portion of the granules forming an electron-lucid core and an outer electron-dense crescent rim. In the present study of endogenous peroxidase (PO) activity of male and female hamster SGs, secretory granules in the acinar cells were studied by DAB cytochemical technique. Individual granules showed bipartite substructure with the PO activity in a positive center core and unreacted lucid rim in both the male and the female acinar cells. Through isolation of granular fractions, the male and the female granules exhibited the same bipartite structure. We also examined the relation between the PO activity and counterstained areas in male and female hamster SGs, and the secretory granules of acinar cells by using EFTEM. In the male SG, the secretory granules exhibited the characteristic bipartite substructure to carry out parallel-EELS, nitrogen reflecting the presence of DAB moieties and uranium from counterstaining the presence the central core but not in the rim. On the other hand, the female bipartite secretory granules of the SG, exhibit the nitrogen reflecting the presence in the central core and uranium in the rim.

Introduction

Many classical studies of variation between the salivary glands of male and female rodents have been conducted. Lacassagne and Fekete (see Shackleford and Klapper, 1962) reported structural differences in the submandibular glands of male and female mice. Junqueira et al. (see Shackleford and Klapper, 1962) have shown biochemical disparities in the mouse submandibular gland. Moreover, the sex of the animals may also modify the fine structure of granules. Seromucous granules of the female hamster submandibular gland have an outer electron-dense area with an electron-lucent larger central area; and an electron-dense crescent located at the periphery of the granules. In the male the reverse is seen, with the larger portion of the granules being electron-dense and the crescent being electron-lucent (Dorey and Bhoola, 1972; Pinkstaff, 1980).

On the other hand, oral antibacterial activity of salivary peroxidase (PO) is widely accepted in the field of dentistry, and PO is known to be a clinically important salivary enzyme (Tenovuo and Pruitt, 1984) and to occur in a variety of glands (Herzog and Miller, 1970; Kataoka et al., 1974; Garrett and Kidd, 1976). Previously, we found PO activity in acinar cells as well as in the granular duct cells of the male hamster submandibular gland (Utsumi et al., 1997). PO reaction products was visible in the cisternae of the endoplasmic reticulum, including in the nuclear envelope and secretory granules. In particular, the secretory granules formed characteristic bipartite structures. The purpose of this investigation to determine whether PO differences between the sexes could be detected by EFTEM cytotochemistry.

Materials and Methods

Five 3-month-old male and female golden Syrian hamsters (CKS, Nagoya, Japan) were used in the present study. They were kept in standard laboratory conditions of a light-dark schedule and relative humidity, and kept with free access to food and water. Under deep anesthesia with an interperitoneal injection of sodium pentobarbital (50 mg/kg body weight), the submandibular glands of
The experimental protocol of this study followed the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. Some specimens were fixed in a mixture of 2% paraformaldehyde (pFA)-2% glutaraldehyde (GA) in phosphate-buffered saline, pH 7.4 (PBS), at 4°C for 60 min. Fixed blocks were washed in the same PBS for 10 min. Then 40-µm-thick sections were prepared using a Leica Cryo-cut 1800, and these sections were rinsed and kept overnight in cold PBS. The sections were incubated in the reaction medium (Utsumi et al., 1997; Moriguchi et al., 1995; Moriguchi et al., 2000), containing 0.1% 3,3’-diaminobenzidine-tetrahydrochloride (DAB) (Dojindo Pharmaceutical Chemical Lab, Kumamoto, Japan) and 0.01% H2O2 in PBS. The incubations were carried out at 37°C for 60 min, followed by post-fixation at 4°C for 60 min in PBS containing 1% osmium tetroxide, dehydration in graded ethanol solutions, and embedding in Quetol 653 resin (Nisshin EM, Tokyo, Japan). Ultrathin sections were cut on a LKB ultramicrotome and examined using a JEM-1210 electron microscope (JEOL, Tokyo, Japan) without uranyl acetate and lead citrate counterstaining. Other 2% pFA-2% GA fixed specimens were post-fixed at 4°C for 60 min PBS containing 1% osmium tetroxide without DAB reaction medium, dehydrated in graded ethanol solutions, and embedded in Quetol 653 resin. Ultrathin sections were examined with counterstaining using an electron microscope.

To obtain extracts with significant granular pellets, we used six parts of the male and female hamster submandibular glands. These glands were immediately removed and separated from the sublingual glands. The glands were dissected free from connective tissue, minced with razor blades into small fragments, and homogenized as a 5% at suspension in a glass potter with a fitted Teflon pestle turning at 1500 rpm using a stirring apparatus for three strokes with intervals of 1 min in the ice bath. The homogenizing medium consisted of 340 mM sucrose and 0.5 mM ethylenediaminetetraacetic acid (EDTA) in 10 mM HEPES buffer, pH 7.4. The homogenate was filtered through four layers of clean cheese cloth to remove fibrous connective tissue and insoluble particles. A pellet of the crude fraction containing secretory granules (P2) was obtained by fraction centrifugation. To obtain the granular fraction, the P2 was resuspended in 25 ml of the homogenizing medium and vacuum filtered successively through 8, 5 and 3 µm Millipore filters (Membrane Filters, Advantec, Tokyo Roshi Kaisha, Ltd., Japan). The filtrate was centrifuged at 18,000 rpm (36,800 ×g) for 30 min to produce a granule-rich fraction (FP2). All centrifugation and filtering steps were performed at 0–4°C (Borges-Silva and Bento-Alves, 1996). Each FP2 was fixed in the mixture of 2% pFA-2% GA in PBS at 4°C for 60 min. Some fixed fractions were washed in the same PBS for 10 min, and were rinsed and kept overnight in cold PBS. The fractions were incubated in the DAB reaction medium (Utsumi et al., 1997; Moriguchi et al., 1995; Moriguchi et al., 2000), containing the 0.1% DAB and 0.01% H2O2 in PBS. The incubations were carried out at 37°C for 60 min, followed by post-fixation at 4°C for 60 min in PBS containing 1% osmium tetroxide, with dehydration in graded ethanol solutions and embedding in Quetol 653 resin. Ultrathin sections were examined by electron microscope without counterstaining. Other fixed fractions were post-fixed at 4°C for 60 min in PBS containing 1% osmium tetroxide without DAB reaction, and dehydrated in graded ethanol solutions and embedded in Quetol 653 resin. Ultrathin sections were examined by electron microscope with counterstaining.

Following the DAB reaction procedures mentioned above, ultrathin sections were also counterstained with uranyl acetate and lead citrate. To carry out Parallel-EELS analysis of the nitrogen reflecting the presence of DAB moieties, (DAB reaction solution, consisting of 0.1% DAB, 0.01% H2O2), we used the Carl Zeiss LIBRA 120 EFTEM (Oberkochen, Germany) operated at 120 kV. To analyze parallel-EELS, a slow scan CCD camera linked to a computer recorded energy-filtered images of identical elements. Parallel-EELS analysis of uranium was also performed.

Results

Ultrastructure of male and female granules

After fixation with glutaraldehyde followed by osmium tetroxide postfixation of the acinar cells of the male hamster submandibular gland, secretory granules exhibited the characteristic bipartite substructure, with the electron-lucid rim and the more dense central core. The ultrathin sections were counterstained with uranyl acetate and lead citrate (Fig. 1A). In females, the reverse was seen, with the larger central portion of granules being electronlucent, and the crescent being electron-dense rim (Fig. 1B), with counterstaining.

PO cytochemistry of male and female granules

Reaction products of PO (the DAB reaction solution, consisting of 0.1% DAB, 0.01% H2O2) in the male and the female secretory granules exhibited the bipartite structure without counterstaining. Both male (Fig. 2A) and female (Fig. 2B) bipartite granules exhibited the PO reaction positive core and lucid rim positions.

PO cytochemistry of isolation granular fraction in male and female

The FR2 of secretory granules obtained from the acinar cells in the submandibular gland. The PO reaction products of the male and the female granules exhibited bipartite structure without counterstaining. Both male (Fig. 3A) and female (Fig. 3B) granules exhibited the PO reaction positive core and lucid rim positions.
Fig. 1. Electron micrograph of hamster submandibular gland specimen fixed with glutaraldehyde followed by osmium tetroxide postfixing. Ultra-thin sections have been counterstained with uranyl acetate and lead citrate. Males: the secretory granules exhibit bipartite granules with the electron-lucid rim and a denser central core (A). Females: the secretory granules display a peripheral rim of relatively high electron density (B).

Fig. 2. Submandibular gland showing PO activity. Ultrathin section observed without counterstaining. Both male (A) and female (B) bipartite granules exhibit the PO reaction positive core and lucid rim positions.

Fig. 3. The PO activity shows the secretory granules fraction obtained from the submandibular gland. Ultrathin section are observed without uranyl acetate and lead citrate counterstaining. Both male (A) and female (B) bipartite granules exhibit the PO reaction positive core and lucid rim positions.
Parallel-EELS analysis of PO in male and female granules with counterstaining

The Parallel-ESI analysis showed the distribution of the nitrogen (the DAB reaction solution, peroxidase activity, consisting of 0.1% DAB, 0.01% H₂O₂) as a structural image in the hamster submandibular gland, and the granules of the acinar cells were present in ultrathin sections counterstained with uranyl acetate and lead citrate (Fig. 4). Parallel-EELS analysis show the distributions of nitrogen and uranium in the same granule. The distributions of nitrogen peak (arrow N) correspond with those of the nitrogen peak (arrow U) in the male granules (4A), but not with those of the female granules (4B).

Discussion

Classically, sialomucin sex dimorphism is evident in the submaxillary and submandibular glands of hamsters. In adult females, alcian blue staining is more intense and the photometric measurement of sialic acid is almost twice that of the adult male. The higher concentration of sialic acid substance probably accounts for the more intense alcian blue reaction in paraffin sections of the female submandibular gland (Shackleford and Klapper, 1962). The histochemical demonstration of tryptophan and tyrosin in the submandibular gland of the albino hamster reveal def-

Fig. 4. Following the DAB reaction procedures, male and female ultrathin sections have been counterstained with uranyl acetate and lead citrate. Parallel-EELS analysis show the distributions of nitrogen and uranium in the same granule. The distributions of nitrogen peak (arrow N) correspond with those of the nitrogen peak (arrow U) in the male granules (4A), but not with those of the female granules (4B).
inite differences according to sex. These differences vary with age. The response in the male is greater than that in the female in these instances (Kronman, 1963b). Moreover, histochemical variations in acid phosphatase activity according to sex and age have been established in the submandibular and sublingual glands of the albino hamster. At all stages in which these differences have been demonstrated, acid phosphatase activity is more pronounced in the female. Maximum variation of reaction has been noted at 3 and 6 months of age, with lesser disparity in the earlier and later stages. Species differences in salivary gland reactivity have been discussed (Kronman, 1963a). In terms of other cytochemistry, we found PO activity in the acinar cells as well as the granular duct cells in the male hamster submandibular gland (Utsumi et al., 1997). We then observed the postnatal developments of the hamster submandibular gland, with granules in the acinar cells showing considerable variations in size, shape, and electrondensity of the PO by light and electron microscopy. In this case, the results have shown that the secretory granules of the hamster submandibular gland undergo changes of area and intensity in the PO activity during the 6 months after birth (Utsumi et al., 2010).

On the other hand, after glutaraldehyde fixation and postfixation in osmium tetroxide, the granules exhibit a bipartite structure at the electron microscopic level. In the
gland of the male hamster, the granule substructure is often fairly typical in appearance with a dense center region and a fairly lucid crescent rim. Dorey and Bhoola (1972) have reported similar observations. In the female, the reverse is seen, with an electron-lucent area and a crescent located at the periphery of the granules. In human salivary glands (Tandler and Erlandson, 1972) as well as in the glands of several other species, granule structure shows great morphological variation; in addition, the ultrastructural appearance of the secretory granules of the human submandibular gland is strongly influenced by fixatives employed in electron microscopy (Tandler and MacCallum, 1972; Tandler and Erlandson, 1972). After fixation in glutaraldehyde, the granules remain unaffected and retain all their protein. When osmium tetroxide alone is employed as fixative, the granule matrix is extracted to a large degree, and granule shape is highly distorted. In this study, the secretory granule fractions were obtained from the submandibular gland. Reaction products of the PO in the male and the female granule fractions exhibited the same bipartite structure similar to those observed in whole submandibular glands of the both sexes, as mentioned above, without counterstaining.

In the present investigation, we also examined the relation between the PO activity and counterstaining areas of the granules in the male and the female acinar cells of the hamster submandibular gland using EFTEM to visualize DAB reactions, with ultrathin sections counterstained with uranyl acetate and lead citrate. In male submandibular glands, the secretory glands exhibit the characteristic bipartite substructure to carry out parallel-EELS, with the nitrogen reflecting the presence of DAB moieties and the uranium from counterstaining in the central core but not in the rim. On the other hand, the female submandibular gland exhibits nitrogen, reflecting its presence in the central core and uranium in the rim. These male and female discrepancies may be readily explained on the basis of sexual differences. Tandler et al. (1999) proposed granule substructure by postulating a self-assortment mechanism for the different molecules in the granule matrix, leading to the production of patterning based on distribution of electron-dense components. Some experimental evidence backs this supposition (Takano et al., 1991; Takano et al., 1993), whereas any clear conclusions have not yet been reached.

Acknowledgments

This study was supported by a Grant-in-Aid for Science Research (1001) grant from the Ministry of Education, Science, Culture and Sports, Japan.

References