IMMUNOHISTOCHEMICAL LOCALIZATION OF EPIDERMAL
GROWTH FACTOR IN THE INTERCALATED DUCT
SECRETORY GRANULES OF THE SUB-
MANDIBULAR GLANDS OF ADULT
FEMALE A/J MICE

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The unlabeled antibody peroxidase-antiperoxidase method and the protein A-gold colloid method were used for localization of EGF in the intercalated duct secretory granules of the submandibular glands of adult A/J "female" mice. Immunoreactive EGF was detected in the secretory granules of the intercalated duct cells and granular convoluted tubule cells. Electron microscopically, EGF was localized only in the secretory granules with the gold particles, indicative of EGF not being distributed homogeneously over the granules. A characteristic finding was an EGF-positive "peripheral part" and an EGF-negative "central part". However, the density of labeling by gold particles over the peripheral part was lower than that within the secretory granules of the granular convoluted tubule cells. The findings suggest that the intercalated duct secretory granules are only a minor source of EGF in the submandibular glands of adult female mice.

Cohen (2) first isolated epidermal growth factor (EGF) from the salivary glands of adult male mice. Since then, many investigators (4, 13) using immunofluorescence or immunohistochemistry have detected the presence of EGF only in secretory granules of the granular convoluted tubule (GCT) cells in the mouse submandibular glands. On the other hand, radioreceptor assay reveals very minute quantities of mouse EGF in other tissues such as kidney, liver and parotid gland (3) but immunocytochemical techniques fail to demonstrate the EGF in the above tissues except for submandibular glands. Therefore, a unit source of EGF at the histochemical level appears to be only secretory granules of the GCT cells in the mouse submandibular glands. We have however a speculation that EGF might be produced in other part of the submandibular glands and therefore focus on the intercalated duct (ID) secretory granules as the other part of EGF production. The present study indicates both the additional localization of EGF in the submandibular glands and a part of the enzymic nature of the ID secretory granules.

MATERIALS AND METHODS

As the submandibular glands of the A/J "female" mice are known to contain high levels of EGF (12), this strain was chosen to investigate the cellular and sub-cellular localizations of EGF in the intercalated duct (ID) cells of submandibular glands. The mice were perfused through the heart with a cacodylate-2% para-formaldehyde-2.5% glutaraldehyde solution (pH 7.4), containing 4% sucrose at room temperature. The tissues were removed, minced, washed in cacodylate buffer, dehydrated in ethanol and embedded in Spurr's resin.

The immunocytochemical procedures for EGF and controls were processed as previously described (5, 11). In brief, for the light microscopic immunocytochemical localization of EGF, resin semithin sections (1 μ m in thickness) were mounted on glass slides, dried on a hot plate (60–70°C) and immersed for 20–40 sec into ethanol saturated with NaOH to remove the resin. They were then very gently rinsed in tap water and were again dried on the hot plate. Finally, the sections were stained by the unlabeled antibody method of Sternberger (9) using 1 : 100 dilutions of the anti EGF serum (Collaborative Research, Inc. Mass. USA). For the electron microscopic immunocytochemical localization of EGF, thin sections, mounted on nickel grids, were stained by the protein A-gold colloid method (7, 10). The primary antisera : anti EGF serum (1 : 500) were applied overnight at 4°C and protein A-gold colloid (gold particle size: 10–15 nm, E. Y. Laboratories, Inc., Calif., USA or 15 nm, Janssen Pharmaceutica, Beerse, Belgium) for 1 hr at room temperature. As controls, (1) DAB reaction only was employed to check the presence of endogenous peroxidase in the submandibular glands; (2) anti EGF serum was replaced by normal rabbit serum (1 : 100 or 500) or (3) the antiserum preabsorbed with an excess of EGF (E. Y. Laboratories, Inc., Calif., USA).

RESULTS

Fig. 1 reveals a portion of the submandibular glands consisting of acini, intercalated ducts and granular convoluted tubules. Some ID cells are located immediately adjacent to the acini and contain immunoreactive material. In addition, all granules of the GCT cells also contain immunoreactive materials. In the longitudinal section, the ID cells appeared cuboid or flat, and a few of them contained EGF-positive granules. Other ID cells with EGF-positive granules, which were probably cut in cross section, were observed in the vicinity of the ductal lumens.

Figs. 2 and 3 are light micrographs of semithin sections stained by the DAB solution only to check endogenous peroxidase (control 1) or the normal rabbit serum (control 2), respectively. Neither the secretory granules of GCT cells nor those of the ID cells revealed EGF-positive reaction. Semithin sections stained by the antiserum preabsorbed with an excess of EGF (control 3) were also EGF-negative (not shown).

At the ultrastructural level using the protein A-gold colloid method, the GCT cells contain many secretory granules of various sizes. Homogeneous distribution of gold particles indicative of EGF was noted over the secretory granules, but no organelle except for the secretory granules was labeled with the gold particles (Fig.4). On the other hand, in the ID cells (Figs. 5, 5a), which contain numerous

FIG. 1. A light micrograph of the submandibular glands of a female A/J mouse stained immunocytochemically for EGF. GCT cells (G) and four ID cells (Nos. 1-4) contain EGF-positive secretory granules. Two cross sectioned ID cells (Nos. 1 and 4) face round ductal lumens (thick arrows) and an ID longitudinally sectioned, which locates between ID cells (Nos. 1 and 3) consists of flattened cuboidal cells. The cells contain a few EGF-positive granules (thin arrows). Acinar cells (A). Unstained $\times 1,200$

FIG. 2. A light micrograph of the glands of a female A/J mouse stained by DAB solution only. No immunoreactive product is seen. Granular convoluted tubule (G). Unstained $\times 330$

FIG. 3. A light micrograph of the glands of a female A/J mouse whose antiEGF serum was replaced with normal rabbit serum. The secretory granules of both GCT cells (G) and ID cells (arrow) are immunoreactively negative (compare with the secretory granules in Fig. 1.). Unstained $\times 1,000$

secretory granules of varying size and density as shown by Caramia (1), the diameter of most secretory granules was less than $1 \mu\text{m}$, and the electron density in the peripheral parts of the secretory granules was higher than that in their central parts (see pentagrams in Figs. 5 and 5a). The distribution of gold particles over the secretory granules, unlike that over the secretory granules in the GCT cells, was not homogeneous, in other words the particles were mostly localized in the peripheral electron-

FIG. 4. An electron micrograph of a cytoplasmic portion of a GCT cell. Gold particles indicative of EGF are homogeneously localized over most secretory granules (S) except for small granules (arrow) labeled with a few particles, but nucleus (N), mitochondria (M) and rough endoplasmic reticula (ER) are never labeled with gold particles. Unstained $\times 26,000$

dense parts, and the number of these particles over each granule was almost unchangeable and was much fewer than that over each granule in the GCT cells (compare Fig. 4 with Fig. 5), suggesting a reduced amount of EGF in the ID secretory granules.

DISCUSSION

Gresik and Barka (5) and Steidler and Reade (8) first reported that the secretory granules in the GCT cells are a major source of EGF, and the secretory granules-containing GCT cells are more abundant in the submandibular glands of adult male mice than those of adult female mice. Caramia (1) observed also that only female submandibular glands of adult mice are characterized by the presence of the secretory granules in the ID cells, but the ID cells in male glands do not contain the secretory granules at all. Judging from our findings in addition to the above reports, the localization of EGF in the ID secretory granules could be explained as follows. In the submandibular glands of adult female mice, the GCT cells are too poorly developed compared with those in males to supply the adequate amount of EGF required in their life, so that the EGF in the ID cells might be necessary to supplement the insufficient EGF production in the GCT cells of adult female mice. Little

FIGS. 5 and 5a. Electron micrographs of a cytoplasmic portion of an ID cell. Gold particles indicative of EGF are dispersed at the peripheral parts of most secretory granules but are not seen in their central parts (arrows), and their peripheral parts are often electron-dense (pentagrams). Nucleus (N). Fig. 5. Unstained $\times 31,000$, Fig. 5a (inset). stained with uranyl acetate only. $\times 50,000$

is known of the enzymic nature of ID cells, particularly in regard to the enzymes present in the secretory granules of the duct cells. In an immuno-fluorescence study of the human parotid gland, Kraus and Mestecky (6) indicated the presence of immuno-fluorescence based on amylase in the ID cells, but there is still a question as to whether this study at the light microscopic level reveals true localization of amylase in the ID secretory granules. However, the present study at light and electron microscopic levels clearly demonstrated that at least the ID secretory granules in the submandibular glands of adult female mice contain EGF. Furthermore, these granules might contain not only EGF but also renin, proteases and nerve growth factor, because Tanaka *et al.* (11) reported that most secretory granules in the GCT cells simultaneously contain both EGF and renin.

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