IMMUNOHISTOCHEMICAL LOCALIZATION OF EPIDERMAL GROWTH FACTOR IN RODENT SUBMANDIBULAR GLANDS

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Histochemically detected distribution of epidermal growth factor (EGF) in submandibular glands (SMG) of the mouse, rat, hamster, and guinea pig is reported using horseradish peroxidase-labeled antibody method. The following main points were established:

1. EGF in the SMG was confined to the granular convoluted tubule (GCT) cells and duct system.
2. EGF reaction in the GCT was most intense in the male mouse.
3. Sexual dimorphism of EGF distribution was very evident in mouse SMG, and somewhat so in rat and hamster glands, and not apparent in guinea pig glands.
4. EGF reaction in mouse SMG increased during pregnancy and was high at 1 and 2 weeks after delivery.
5. EGF in the postnatal stage of male mouse first developed at 3 weeks after birth.

It has been reported that higher amounts of biologically active polypeptides: epidermal growth factor (EGF) and nerve growth factor (NGF) as well as proteases and renin are confined to the granular convoluted tubule (GCT) cells in the submandibular gland (SMG) of mice (1, 9, 13, 18, 28, 30). GCT cells of the SMG are particular structures in mice and rats and have responded extremely well to administration of androgen and thyroxine. It has been mentioned that for rodents, the GCT cells are present in mice and rats but not in guinea pigs. Originally, GCT cells in the SMG differentiate from the ductal segment during postnatal development (7, 12, 25, 26). At birth, the SMG consists of terminal tubules and the gland shows no sex difference. There are reports (6, 12) that cytodifferentiation from terminal tubules to ductal segments including the GCT and striated duct occurs approximately 3 weeks after birth in mice and rats.

The present study carried out EGF reaction in the GCT cells during postnatal development in mice SMG of both sexes and also determined the EGF distribution of SMG in pregnancy and postpartum stages.

EGF was first isolated from the mouse SMG, and immunological cross reaction then noted between mouse and rat (11). Many reports of immunohistochemical demonstrable EGF and radioimmunoassay EGF have been described in mouse specimens and a few times in rats, but to reports have been made of materials from
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the SMG of hamsters, or guinea pigs. The present experiments were immunohistochemical detection of EGF in SMG of hamsters and guinea pigs as well as mice and rats of both sexes, and were compared to mice specimens.

MATERIALS AND METHODS

1. Isolation of EGF
   a. Gel chromatography:
      SMGs were obtained from 50 adult male ddy mice, and the method of EGF isolation from the mSMG used was a modification of the Savage and Cohen technique (23). The material applied to the Bio-Gel p-10 at 4°C was collected using a LKB fraction collector. The elution pattern obtained from the chromatography showed seven peaks, designated as p-1 to p-7 (Fig. 1).
   b. Immunodiffusion:
      Double immunodiffusion was used for the detection of EGF, with anti-EGF serum being obtained from Collaborative Research, U.S.A. All fractions were assayed by double immunodiffusion, and EGF was present mainly to the p-7 fraction (Fig. 2).
   c. Iso-electric focusing:
      An ampholine column (110 ml) with a sucrose gradient (15-50%) and gel filtration material was prepared using a gradient mixer (Pharmacia Fine Chemicals). Iso-electric focusing was conducted for 16 hr at 1200 volts. The fractions were neutralized to pH 7.0 with 1 N NaOH and dialyzed for 36 hr. The p-7 fraction was dialyzed and fractionated by isoelectric focusing. From this pattern, the fraction with a peak at pH 4.5 was neutralized to pH 7.0 with 1 N NaOH solution and dialyzed.
   d. Disc electrophoresis:
      For testing purification of the pH 4.5 peak in p-7 fraction, disc electrophoresis

![Fig. 1. The elution pattern from the acidic Bio Gel P-10 chromatography showed 7 peaks, designated as p-1 to p-7.](image-url)
FIG. 2. Ouchterlony pattern for mEGF, center: anti-mEGF serum (Collaborative Research), a: p-1, b: p-4, c: p-6, d: p-7. Interaction between the d well (p-7) and center well (anti-mEGF) is most intense.

FIG. 3. Single band of disc electrophoresis in p-7 fraction.

of 10% polyacrylamide gel was carried out (Fig. 3). Single band was obtained from Auto Scanner FLUR-VIS, Helena Laboratories.

e. Bioassay:
For testing biologic activity in the p-7 fraction, a bioassay was employed. Three groups of newborn mice (ddy) were administered subcutaneous daily injections of either 10, 5, or 2.5 μg EGF/g weight. Two groups of newborn hamsters were also subcutaneously administered daily injections of either 5 or 1 μg/g weight.

2. Immunohistochemical method of EGF localization

a. Anti-EGF serum:
Adult rabbits were injected subcutaneously 3 times at 10 day intervals with 3 mg
of isolated EGF in 1.5 ml phosphate buffered saline (PBS) to which was added an equal volume of Freund’s complete adjuvant. One week after the last injection, whole blood was obtained from the carotid artery, and the serum was separated and used as a source of anti-EGF. A part of the anti-EGF serum was purchased from LAREF SA, 6814 Cadempio, TI, Switzerland.

b. Preparation of horseradish peroxidase-labeled antibody:

The method of Nakane & Kawaoi (19) was used to prepare horseradish peroxidase-labeled antibody. Goat anti-rabbit IgG antiserum was conjugated with horseradish peroxidase (Sigma). The conjugate was filtered through a Sephadex G-200 column and the first peak was used as horseradish peroxidase-labeled antibody.

c. Immunohistochemical method:

i) Specimens: SMGs obtained from adult ddy mice, rats (Wistar), hamsters and guinea pigs of both sexes were employed. SMGs from pregnant mice, both before and after delivery, were also used to examine fluctuations in EGF levels throughout and following pregnancy. The SMG of newborn mice and those 1, 2, and 3 weeks postpartum were employed to detect EGF during postnatal development.

ii) Tissue preparation: Small pieces of SMG were fixed in Bouin solution overnight at 4°C and rinsed in PBS for 24 hr. The fixed tissue was then dehydrated through an alcohol series and embedded in paraffin. 6 μm serial sections were made.

iii) Immunohistochemical staining: To inactivate endogenous peroxidase (4, 8), deparaffinized sections were treated with 0.3% H₂O₂/methyl alcohol solution at room temperature for 20 min and rinsed in PBS for 20 min. The sections were then treated with rabbit anti-mouse EGF (mEGF) serum for 60 min and rinsed in PBS for 60 min, after which they were made to react with horseradish peroxidase-labeled goat anti-rabbit IgG for 30 min and rinsed in PBS for 30 min. Finally, the sections were incubated in diaminobenzidine (DAB)/H₂O₂ solution for 10 min at room temperature and rinsed with PBS for 15 min. Areas positive for EGF were stained dark brown.

iv) Control experiment: The following control test in SMG was carried out.

1) The sections were incubated with normal rabbit serum instead of rabbit anti-mEGF. 2) The sections were incubated with rabbit anti-mEGF (1 : 15) added with purified mEGF (100 μg/ml). The incubation media was kept overnight by mixing anti-mEGF and mEGF at 20°C. 3) These sections were immersed in DAB/H₂O₂ solution for 10 min at room temperature.

3. Histological and histochemical methods

Histologic orientation of animal SMG was done by hematoxylin eosin stain, and toluidine blue stain following 10% formalin fixation. Dimethylaminobenzaldehyde (DMAB) stain for tryptophan was carried out for comparing EGF reactions.

RESULTS

1. Bioassay of EGF

The mice injected with the p-7 fraction opened their eyes and showed tooth eruption on the 8th day in all groups. Control mice opened their eyes on the 12th day and showed tooth eruption on the 10th day. The hamsters opened their eyes
on the 10th day in the 5 μg/g group and on the 13th day in the 1 μg/g group. Control hamsters opened their eyes on the 15th day. No examination for tooth eruption was made, because newborn hamsters have incisor eruption at birth.

Figs. 4 and 5. EGF in adult mouse submandibular gland (SMG). Fig. 4 male and Fig. 5 female. EGF is limited to the granular convoluted tubule (GCT) cells. Figs. 4 and 5 × 200

Figs. 6 and 7. DMAB staining in adult mouse SMG. Fig. 6 male and Fig. 7 female. DMAB positive materials in the GCT cells are higher in male than in female. Figs. 6 and 7 × 200