Demonstration of significant epidermal growth factor activity in murine thyroid tissue

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ABSTRACT

Epidermal growth factor (EGF) was first isolated from the mouse submaxillary gland (SMG) which remains its only known site of synthesis. Earlier work suggests that EGF may be implicated in the regulation of thyroid cell growth and proliferation. Moreover, thyroid hormones increase the EGF content of mouse SMG and mimic the maturational effects associated with EGF. The aim of this study was therefore to determine whether the mouse thyroid gland is a site of EGF production. Thyroid and submaxillary glands were homogenized in Tris-HCl, the EGF content was measured by a homologous radioimmunounassay (RIA) and receptor-binding activity assessed in a radioreceptor assay.

Epidermal growth factor was readily detectable in each thyroid extract. Dilution curves were parallel to the standard curve. Values obtained for right and left lobes of thyroid were in excellent agreement ($r = 0.997$, $P<0.001$). The intrathyroidal EGF concentration obtained by RIA was $26.1\pm6.0$ (s.e.m.) ng/mg protein ($n=40$); values obtained by the receptor assay were slightly lower but correlated closely ($r=0.828$, $P<0.01$). Assay of homogenates prepared at the same time from the submaxillary glands of these mice showed that thyroidal EGF was not correlated with EGF content in the SMG.

These findings indicate that EGF, both biologically active and immunoreactive, is readily detectable in the mouse thyroid and that the thyroid gland is a probable site of EGF synthesis. The methodology provides a model for further studies of the regulation of EGF production and its significance in relation to thyroid disease.

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INTRODUCTION

Epidermal growth factor (EGF), a polypeptide mitogen of small molecular weight (6000), causes the proliferation of cultured thyroid cells from a wide variety of species. In some cell-culture systems the mitogenic effect of EGF is accompanied by an apparent inhibition of thyrotrophin-stimulated differentiation processes such as iodide uptake and organification (Cohen, 1962; Roger & Dumont, 1982; Westermark, Karlsson & Westermark, 1983; Dumont, Roger, van Heuverswyn et al. 1984).

In the mouse, the only proven tissue of origin of EGF is the submaxillary gland (SMG), where synthesis is controlled by, among other factors, age, sex and thyroid hormones (Byyny, Orth & Cohen, 1972; Gresik, Schenken, van der Noen & Barka, 1981). However, excision of the SMG does not reduce basal EGF concentrations in mouse body fluids, suggesting the presence of alternative sites of synthesis (Byyny, Orth, Cohen & Doyne, 1974; Perheentupa, Lakshmanan & Fisher, 1985).

Byyny et al. (1972) using radioimmunoassay (RIA) methods, obtained the following concentrations of EGF in various adult male mouse tissues: SMG (1000 ng/mg wet tissue), kidney (0.49 ng/mg), stomach (0.43 ng/mg), pancreas (0.21 ng/mg), testis (0.08 ng/mg) and skin (0.06 ng/mg). The same authors reported that EGF was undetectable (<0.01 ng/mg) in adult mouse lung, brain and pituitary. Female mouse SMG contains about 70-100 ng EGF/mg wet tissue (Byyny et al. 1972; Gresik et al. 1981). There have been no previous reports of EGF in the mouse thyroid gland. The present study was undertaken to
determine whether EGF is present in mouse thyroid tissue and, if so, to examine whether the thyroid gland is a possible site of EGF synthesis.

MATERIALS AND METHODS

Preparation of thyroid and SMG homogenates

Thyroid tissue and submaxillary glands were obtained from male BALB/c mice aged from 14 to 16 weeks, immediately after killing by cervical dislocation. The glands were glass–glass homogenized in Tris–HCl (10 mmol/l, pH 7.4) for 60 s using an electrical blender (TRI-R Instruments, New York, U.S.A.). The homogenates were centrifuged at 15,000 g for 30 min at 4 °C. The supernatants were decanted in small aliquots and stored without preservative at −40 °C. The frozen samples were retrieved the following day, thawed on ice and assayed for EGF. Protein concentrations were determined by the method of Bradford (1976) using Coomassie blue (Pierce Chemical Co., Rockford, IL, U.S.A.).

Preparation of liver membrane receptors

Pregnant rat liver, previously snap-frozen and stored at −70 °C was thawed on ice and weighed after drying lightly on tissue paper. The liver was minced manually and further homogenized in 5 vol. Tris-HCl (10 mmol/l, pH 7.4) for 4 s using a polytron blender (Silverson Machines, London). The homogenate was centrifuged at 800 g for 10 min at 4 °C to sediment nuclei and coarse debris and the supernatant was re-centrifuged at 20,000 g for 20 min. The pellet from the second centrifugation was suspended in Tris-HCl buffer (10 mmol/l) containing NaCl (50 mmol/l) and 0.1% (w/v) bovine serum albumin (BSA), pH 7.4. Protein determination was by the Bradford (1976) method.

Mouse EGF and antiserum

Highly purified mouse EGF was purchased from Bethesda Research Laboratories, Cambridge, Camb. Antiserum (rabbit) to purified mouse EGF was a gift from Dr H. Gregory (ICI, Macclesfield, Cheshire). The specificity of the antiserum has previously been established (Gregory, Holmes & Willshire, 1979). The mouse EGF was used for iodination and as a calibrating standard for both the RIA and the radioreceptor assay (RRA).

Iodination of mouse EGF

Highly purified mouse EGF was iodinated using carrier-free 125I by the iodogen method (Fraker & Speck, 1978). The labelled EGF was purified by gel chromatography on a column (1.6 × 58 cm) of Sephadex G-50 Superfine resin (Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer (0.1 mmol/l) containing 0.1% (w/v) BSA (pH 7.4). Approximately 1 ml (3 min) fractions were collected. The peak of radioactivity corresponding to 125I-labelled mouse EGF eluted at 2.2 × void volume (V0) and incorporated 80–100% of the total radioactivity added. Specific activities of approximately 50 Ci/g were obtained. Labelled mouse EGF was diluted in assay buffer and aliquots were stored at −70 °C, thawed only once and used within 8–10 weeks of preparation. The stored radiolabel was stable over this period.

Preparation of EGF-free mouse thyroid extract

Peptide-free mouse thyroid extract was prepared by charcoal-adsorption as previously described for the preparation of EGF-free saliva (Dagogo-Jack, Atkinson & Kendall-Taylor, 1985). The EGF-free status was confirmed both by direct RIA and by adding a small amount of tracer 125I-labelled mouse EGF to the original extract and assessing recovery. The efficiency of the method was 99.7 ± 0.12% (mean ± 2 × s.d., n = 6).

RIA procedure

The RIA was carried out in phosphate buffer (40 mmol/l) containing sodium chloride (0.15 mmol/l), EDTA (10 mmol/l), 0.5% (w/v) BSA and 0.1% (w/v) thiomersal (BDH Chemicals Limited, Poole, Dorset), pH 7.2. Triplicate determinations were made in 11 × 75 mm plastic tubes. The final incubation volume (0.3 ml) consisted of either mouse EGF standard or mouse tissue extract (0.1 ml), antiserum (0.1 ml) in buffer to give a dilution of 1:48 000 and 125I-labelled mouse EGF (10 000 c.p.m., 20 pg) in buffer (0.1 ml). The dilution of antiserum used gave 20% specific binding of the total radioactivity added in the absence of unlabelled mouse EGF. Control tubes contained buffer (0.2 ml) and radiolabel (0.1 ml). The tubes were mixed and incubated for 24 h at 4 °C. Antibody-bound and free fractions were separated using solid-phase second antibody (Sac-Cel, Wellcome Reagents Limited, Beckenham, Kent). After centrifugation, the supernatant was aspirated and the radioactivity in the pellet counted in a multi-well gamma counter (Wilj Gambyt CS-20). The results were calculated using a standard RIA computer program based on log transformed data.

Receptor binding assay

The standard diluent was Tris–HCl buffer (10 mmol/l) containing sodium chloride (50 mmol/l) and 0.1%
Liver membrane preparations were diluted in buffer such that a 100 \( \mu l \) aliquot/tube gave a final membrane protein concentration of 1 mg/ml. Each assay tube received 0.2 ml of either serially diluted mouse EGF standard or mouse tissue extract, 0.1 ml liver membrane suspension and 0.1 ml \(^{125}\)I-labelled mouse EGF dilution (15000 c.p.m., 30 pg). An excess (3 \( \mu g \)) of unlabelled mouse EGF was added to triplicate control tubes to give an indication of non-specific binding. The tubes were incubated for 90 min at 26 \(^\circ\)C with continuous agitation, the reaction was terminated by the addition of 1 ml ice-cold buffer and bound and free moieties were separated by centrifugation (10 000 g for 20 min at 4 \(^\circ\)C). The supernatant was aspirated and the radioactivity in the pellet counted. Specific binding was defined as the difference between the radioactivity bound in the assay tubes and that in the control tubes containing excess mouse EGF.

All measurements were carried out in triplicate and results are expressed as means \( \pm \) s.e.m. The significance of differences in mean values between groups was assessed by Student's \( t \)-test and the correlation coefficient (\( r \)) was determined to assess the significance of the correlation between mouse EGF concentrations in defined groups.

**RESULTS**

**Immunoreactive mouse EGF**

Mouse EGF was readily detectable by the RIA in extracts from the SMG and thyroid in each mouse studied. Figure 1 shows a representative dose–response curve for the mouse EGF RIA compared with the curves generated by dilutions of thyroid and SMG homogenates. Analysis of variance on the slopes (unweighted least squares linear regression) of the thyroid and SMG dilution curves and of the slope for three points in the mid-portions of the standard curve did not show significant deviations from parallelism. The standard curves obtained using either peptide-free pooled thyroid extract or phosphate buffer as diluent were superimposable (data not shown).

The limit of detection, defined as the concentration of unlabelled mouse EGF that resulted in two standard deviations from the initial binding in zero standard tubes, was 40.32 \( \pm \) 3.05 pg/tube (22.4 \( \pm \) 5.1 pmol/l) over 12 consecutive assays.

Thyroid extracts from three mice were assayed for mouse EGF a number of times within the same batch. The mean (\( \pm \) s.d.) mouse EGF concentrations (pmol/l) were 316.5 \( \pm \) 25.3 (\( n = 10 \)), 413.5 \( \pm \) 53.5 (\( n = 9 \)) and 1036.7 \( \pm \) 168.5 (\( n = 7 \)). The coefficients of variation were 7.9, 12.9 and 16.3\% respectively. Three solutions of mouse EGF standard were prepared in buffer to give concentrations of 164, 410 and 1640 pmol/l respectively. These were stored at \(-40 \, ^\circ\)C in aliquots and assayed repeatedly for mouse EGF over 12 consecutive assays. The means (\( \pm \) s.d.) were 158.4 \( \pm \) 22.1, 441.8 \( \pm \) 45.0 and 1478.3 \( \pm \) 175.6 pmol/l respectively. The coefficients of variation were 13.9, 10.2 and 11.9\% respectively. The level of non-specific binding either in buffer or in charcoal-adsorbed mouse extract was less than 2\% of the total binding. The rabbit antiserum to mouse EGF did not cross-react in this radioimmunoassay with a variety of peptide hormones including human EGF (164–8200 pmol/l).

The mean concentration of immunoreactive mouse EGF in thyroid homogenates was 26.1 \( \pm \) 6.0 ng/mg protein, with a range from 5.5 to 68.0 ng/mg protein. Submaxillary gland extracts contained mouse EGF in concentrations (600–1220 ng/mg wet tissue or 13.0–27.0 \( \mu g \)/mg protein) that were considerably higher than those found in the thyroids.

**Receptor-assayable mouse EGF**

Figure 2 shows a typical dose–response curve for the mouse EGF RRA assay and compares the curves given by dilutions of thyroid and SMG extracts.
The mouse EGF detectable by RRA was somewhat lower than that detectable by RIA but the difference was not significant. The two values for individual extracts were in good agreement (Fig. 3).

Mouse EGF in separate thyroid lobes

To examine whether methodological factors may account for the wide variability observed in intrathyroidal EGF, the right and left lobes of the thyroid were carefully resected from eight mice. The dissection was aided by the use of an angle-poise lamp fitted with a magnifying lens. Each lobe was homogenized separately as previously described and the supernatant applied to the RIA. The result showed that individual variations persisted despite excellent correlation between EGF concentrations in right and left lobes (r = 0.997, P < 0.001).

Simultaneous assay of thyroid and SMG extracts

To determine whether intrathyroidal EGF in the individual mouse was a function of the concentration of EGF in the SMG, homogenates of thyroid and submaxillary glands from 20 mice were simultaneously prepared and assayed. The results, depicted in Fig. 4, showed no correlation between the two glands regarding their EGF content.

**Figure 2.** Dose–response curves for purified mouse epidermal growth factor (EGF) standard (solid line) and dilutions of thyroid (broken lines) and submaxillary gland (dotted line) extracts in the radioreceptor assay. $B$ = fraction of total $^{125}$I-labelled EGF specifically bound to liver membrane receptors. $B_o$ = fraction of total radioactive bound in the absence of unlabelled mouse EGF.

**Figure 3.** Correlation between values for epidermal growth factor (EGF) concentration in mouse thyroid homogenates obtained by radioimmunoassay (RIA) and radioreceptor assay (RRA). There was a strong positive correlation between these two values ($r = 0.828$, $P < 0.01$).

**Figure 4.** Comparison of epidermal growth factor (EGF) concentrations in homogenates simultaneously prepared from the thyroid and submaxillary glands of adult male BALB/c mice.
DISCUSSION

This study demonstrates the readiness with which EGF can be detected both by RIA and RRA in extracts from mouse thyroid and submaxillary glands. The observed range (600–1220 ng/mg wet tissue) of EGF concentrations in the submaxillary glands of adult male BALB/c mice agrees fairly well with the value of 1000 ng/mg quoted for Swiss Webster Albino mice (Byyny et al. 1974) but there are no appropriate previous figures with which to compare our thyroid EGF data. It is noteworthy, however, that the intra-thyroidal levels of EGF observed in this study appear to be several times higher than the figure previously reported for other mouse tissues except the submaxillary glands. A mean thyroid EGF concentration of 26 ng/mg protein (approximately 1·5 ng/mg wet tissue) would be 150 times the value reported by Byyny et al. (1972) in mouse brain and pituitary, and 20 times the concentration in the testes.

The wide variation in thyroid EGF concentrations (5·5 to about 70 ng/mg protein) in our mouse population probably reflects a true trend rather than experimental artifact. The reason for this is not clear, but factors such as metabolic and hormonal differences in the regulation of protein synthesis or degradation may be involved. The injection of thyroxine has been reported to increase (1) the synthesis of EGF in the mouse SMG (Gresik et al. 1981), (2) the accumulation of EGF in neonatal mouse skin (Hoath, Lakshmanan, Scott & Fisher, 1983) and (3) the urinary excretion of EGF in sialoadenectomized mice (Perheentupa et al. 1985).

Besides augmenting EGF synthesis, thyroid hormones may influence EGF receptor numbers (Mukku, 1984) and can induce precocious eye opening and incisor eruption in neonatal mice, two parameters used in the standard bioassay for native EGF (Karnofsky & Cronkite, 1939; Walker, 1957; Hamburgh, Mendoza, Burkart & Weil, 1971; Hoath, Lakshmanan & Fisher, 1983). Thus it is possible that differences in thyroid hormone concentration locally or in the circulation may partly account for the variability seen in mouse intrathyroidal EGF. The lack of correlation between EGF concentrations in the thyroid and submaxillary glands (Fig. 4) in the intact mouse implies that EGF in the thyroid is not simply a reflection of the SMG content. This finding complements the results of previous studies of sialoadenectomized mice, where EGF remained detectable in skin homogenates (Hoath et al. 1983) and in urine (Perheentupa et al. 1985), strongly suggesting the presence of other sources of synthesis.

This study strongly implies the possibility of EGF synthesis in the mouse thyroid. Related findings in man are supportive. Human EGF (urogastrone) is reportedly present in the thyroid gland in greater concentrations (5-3 ng/g tissue) than in any other tissues except the kidney (5-5 ng/g) (Hirata & Orth, 1979). Although both mouse and human EGF are closely related in structure and functions (Cohen & Carpenter, 1975; Gregory, 1975), it is not known whether changes in thyroid EGF content play any role in the pathogenesis of goitre. The methodology which has been established here provides a model for further studies of the regulation of EGF production and its significance in relation to thyroid disease.

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REFERENCES


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