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Human Epidermal Growth Factor: Isolation and Chemical and Biological Properties

(fibroblast growth/competitive binding/urinary polypeptide hormone/corneal epithelium)

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ABSTRACT **A polypeptide hormone has been isolated from human urine, human epidermal growth factor. It was assayed by its ability to compete with ^{125}I-labeled mouse-derived epidermal growth factor in binding to human foreskin fibroblasts. The biological effects of the human polypeptide are similar to those previously described for the mouse hormone. These include the stimulation of the growth *in vitro* of human foreskin fibroblasts and corneal epithelial cells in organ culture, and the *in vivo* induction of precocious eyelid opening in the newborn mouse. The amino acid compositions of the two polypeptides differ, although certain similarities are present. The estimated molecular weight of the human polypeptide, 5300–5500, is slightly lower than that of the mouse hormone. Both polypeptides apparently compete for the same site on the cell membrane; antibodies to the mouse polypeptide crossreact to some extent with the human hormone.**

Epidermal growth factor (EGF) is a single chain, 53 amino-acid-residue polypeptide (molecular weight 6045), that is isolated from adult male mouse submaxillary glands. The complete amino acid sequence and the location of the three disulfide bonds have been reported (1, 2). EGF stimulates the growth of skin and corneal epithelium *in vivo* and in organ cultures (3, 4). In addition, EGF is a potent mitogen for certain human and mouse fibroblasts in culture (5–7). Although mouse-derived EGF (mEGF) is effective with cells from a variety of animals (chick, mouse, and human), its natural presence in species other than the rodent, and thus its general physiological significance, have been in question.

We have recently reported the detection, in human urine, of material with some of the biological and immunological properties of mouse EGF (8). The development of a sensitive radio-receptor competitive binding assay for human epidermal growth factor (hEGF) using human foreskin fibroblasts and ^{125}I-labeled mEGF permitted the isolation of microgram quantities of pure growth factor from protein concentrates of human urine. The isolation procedure and some of the biochemical and biological properties of hEGF are documented in this report.

MATERIALS AND METHODS

Pure mouse EGF and monoiodo ^{125}I-labeled mEGF were prepared as described (9). The ^{125}I-labeled mEGF binding and competition assays were carried out on monolayer cultures of human foreskin fibroblasts (0.8 to 1.2 $\times$ 10^6 cells per 60 mm Falcon dish) by incubating a standard amount of ^{125}I-labeled mEGF in the simultaneous presence of aliquots of competing peptide for 1 hr at 37°. Unbound ^{125}I-labeled mEGF was removed by washing, the cells were solubilized by the addition of 1 ml of 0.5 M NaOH, and the radioactivity was measured with a gamma spectrometer (Nuclear-Chicago). The experimental details of the procedure have been described (9).

Gel filtration and ion exchange chromatography were carried out at 4°. Amino acid analysis was done according to the method of Spackman, Stein, and Moore (10) on a Spinco model 120 C analyzer, equipped with an Infotronics integrator. Performic acid oxidation was carried out according to the method of Moore (11). The apparent weight-average molecular weight was calculated from sedimentation equilibrium data, as reported (12). Solutions of hEGF (A_{280} = 0.36) were made in 0.15 M KCl containing 10 mM potassium acetate buffer, pH 5.6.

The starting material for the isolation of human EGF was an acetone powder of benzoic acid-adsorbed urinary proteins (pregnant women), very kindly supplied by Ortho Diagnostics.

RESULTS

Fibroblast Receptor Assay for EGF. The assay is based on the ability of both mouse and human EGF to compete with ^{125}I-labeled mEGF for binding sites on human foreskin fibroblasts. A typical standard curve obtained by ascertaining the effects of increasing quantities of mEGF (or hEGF) on the binding of a standard amount of ^{125}I-labeled mEGF is shown in Fig. 1. Under these conditions, 2–20 ng of mEGF are readily measurable. In the following experiments, the quantities of hEGF present in the fractions isolated from human urine are expressed as equivalents (by weight) of mouse EGF determined by the competitive binding assay. As previously reported (9), no competition with ^{125}I-labeled mEGF could be detected with a wide variety of known peptide hormones.

Preparation of Human Epidermal Growth Factor. Ten grams of benzoic acid/acetone powder of urinary proteins were suspended in 40 ml of water and adjusted to pH 9 with 1 M NaOH. The mixture was centrifuged at 4° at 18,000 rpm for 10 min and the supernatant collected. The residue was resuspended in 50 ml of cold water and the mixture again centrifuged. The supernatants were combined and lyophilized. This material contained approximately 800 mg of protein [Lowry procedure (13)] and 600 μg of hEGF.

(a) *Gel filtration on Bio-Gel P-10:* The lyophilized powder

Abbreviations: EGF, epidermal growth factor; the letters m or h prefixed to EGF indicate source, mouse or human, respectively.

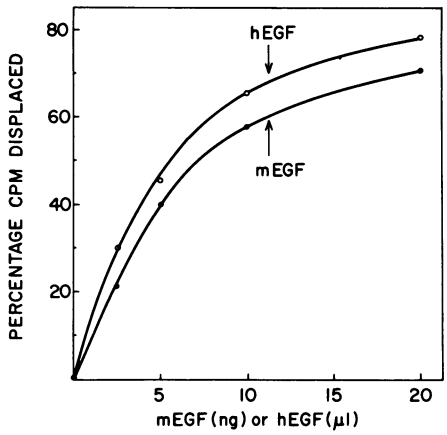

FIG. 1. Competitive fibroblast binding assay for mEGF and hEGF in the presence of ^{125}I-labeled mEGF. The binding medium consisted of 1.5 ml of an albumin-containing, modified Dulbecco medium. Increasing concentrations of mEGF or hEGF were added simultaneously with ^{125}I-labeled mEGF (3.4 ng, 1.2×10^5 cpm) to a monolayer culture of human foreskin fibroblasts (see *Materials and Methods* and ref. 9 for details). Controls contained only the labeled polypeptide and bound approximately 6000 cpm/10^6 cells during the 1-hr incubation period. "Nonspecific" binding, determined by measuring the cell-bound radioactivity in the presence of excess mEGF (15 μg/ml), amounted to less than 3% of the total.

was dissolved in 18 ml of water and centrifuged. The dark brown supernatant solution was applied to a Bio-Gel P-10 column (Fig. 2A). The fractions containing binding activity (between the arrows) were combined and lyophilized. Approximately 80 mg of protein and 550 μg of hEGF were recovered. (It should be noted that the A_{280} values shown in the figures result from both protein and pigment content.)

(b) *Gel filtration on Sephadex G-50:* The lyophilized material derived from the Bio-Gel column was dissolved in 3 ml of water and applied to a Sephadex G-50 column (Fig. 2B). The fractions between the arrows were combined and lyophilized. Approximately 45 mg of protein and 400 μg of hEGF were recovered.

(c) *Passage through DE-52 cellulose, pH 3.0:* The lyophilized fraction after Sephadex G-50 chromatography was dissolved in 11 ml of 0.03 M formic acid (final pH 3.0) and applied to a column of DE-52 cellulose (8.5 × 0.9 cm) equilibrated with 0.03 M ammonium formate buffer, pH 3.0. The column was washed with 40 ml of the same buffer, and the eluate, containing approximately 28 mg of protein and 380 μg of hEGF, was lyophilized.

(d) *Ion-exchange chromatography on CM-52 cellulose:* At this stage of the purification, the lyophilized powders from two of the DE-52 preparations were combined. The sample was dissolved in 16 ml of 0.04 M acetic acid (final pH 3.8–4.0) and applied to a column of CM-52 cellulose equilibrated with 0.04 M ammonium acetate, pH 3.8. After the column was washed with 50 ml of the buffer, a gradient (0.04 M to 2.0 M ammonium acetate, pH 3.8) was applied. The gradient was prepared by allowing 2.0 M ammonium acetate buffer to flow into a 125 ml constant-volume mixing chamber filled with the 0.04 M ammonium acetate solution. A typical elution pattern is illustrated in Fig. 2C. The fractions between the arrows were combined and lyophilized. Approximately 6 mg of protein and 600 μg of hEGF were recovered.

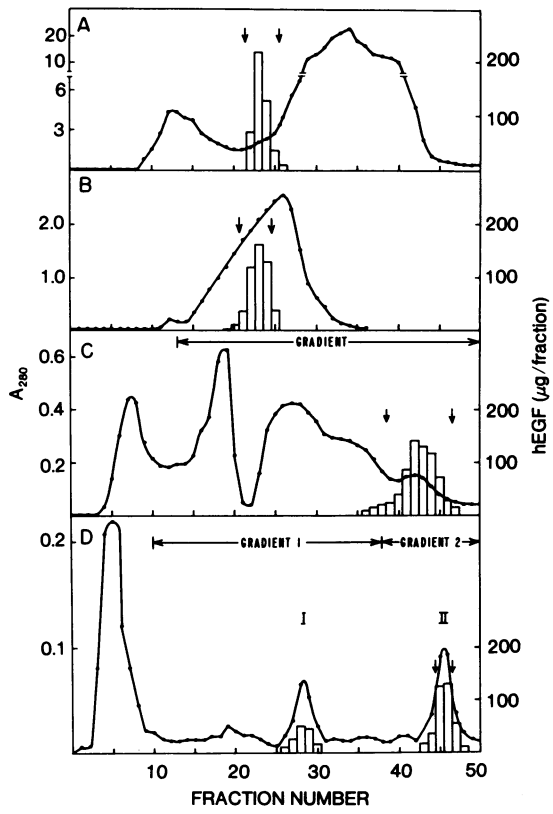

FIG. 2. Gel filtration and ion-exchange chromatography of hEGF. In each figure, the *bar graph* indicates the competitive binding potency of each fraction, expressed as equivalents (by weight) of mEGF. (A) Eighteen milliliters of the extract from 10 g of acetone powder, containing 800 mg of protein, were applied to a Bio-Gel P-10 column (90 × 2.5 cm) equilibrated with 0.01 M ammonium acetate. The flow rate was 28 ml/hr, and 15 ml fractions were collected. (B) Three milliliters of the Bio-Gel fraction, containing 80 mg of protein, were applied to a Sephadex G-50 column (90 × 1.5 cm) equilibrated with 0.01 M ammonium acetate. The flow rate was 11 ml/hr, and 5 ml fractions were collected. (C) Sixteen milliliters of the DE-52, pH 3.0, fraction (two preparations), containing 56 mg of protein, were applied to a column of CM-52 cellulose (10 × 1.5 cm) equilibrated with 0.04 M ammonium acetate, pH 3.8. The protein was eluted with an ammonium acetate gradient (see *text* for details). Five milliliter fractions were collected. (D) Five milliliters of the CM-52 fraction, containing 6 mg of protein, were applied to a column of DE-52 cellulose (8.5 × 0.9 cm) equilibrated with 0.02 M ammonium acetate buffer, pH 5.6. The protein was eluted with two successive gradients of ammonium acetate buffer (see *text* for details). Two and one half milliliter fractions were collected.

(e) *Ion exchange chromatography on DE-52 cellulose, pH 5.6:* The lyophilized powder from the CM-52 chromatographic separation was dissolved in 5 ml of 0.02 M ammonium acetate buffer, final pH 5.6, and applied to a column of DE-52 cellulose equilibrated with the same buffer. The column was washed with 20 ml of the buffer, and then a gradient buffer (0.02–0.2 M ammonium acetate, pH 5.6, using a 65 ml constant-volume mixing chamber) was applied. Seventy milliliters of this gradient sufficed to elute a peak containing binding activity (peak I, Fig. 2D). With the same mixing chamber, a second gradient was applied by permitting 1.0 M ammonium acetate, pH 5.6, to flow into the mixing chamber. A second peak of active material (peak II, Fig. 2D) was thus

TABLE 1. *Amino-acid composition of human epidermal growth factor (apparent residues per mole of protein)*

Amino acid	Native hEGF Exp. 1	Native hEGF Exp. 2	Performic acid oxidized hEGF Exp. 1	Performic acid oxidized hEGF Exp. 2	Probable composition of hEGF	Composition of mEGF
Lys	2.37	1.99	2.69	3.05	3	0
His	1.89	1.55	0.32	0.41	2	1
Arg	1.62	1.81	1.89	1.73	2	4
Asp	7.17	8.20	7.09	6.49	7	7
Thr	Tr	Tr	0.28	Tr	0	2
Ser	3.10	2.86	2.52	3.01	3	6
Glu	5.00	5.00	5.00	5.00	5	3
Pro	1.60	0.82	1.35	1.18	2	2
Gly	3.94	4.83	4.38	3.91	5	6
Ala	1.78	2.08	2.02	1.76	2	0
Half-Cys	1.09	0.93	0.0	0.0	6	6
Val	1.14	0.83	1.90	1.38	2	2
Met	Tr	Tr	0.0	0.0	1	1
Ile	1.03	0.52	1.67	1.28	2	2
Leu	3.78	4.44	4.35	3.80	4	4
Tyr	2.37	1.90	0.25	Tr	2	5
Phe	0.0	0.0	0.0	0.0	0	0
Trp		1.14*			1	2
Cysteic acid	0.42	0.62	5.09	5.54		
Met sulfone	0.0	0.0	0.69	0.86		
			Total residues		49	53
			Molecular weight		5458	6045

Samples of 20–43 μg were hydrolyzed in 6 M HCl under reduced pressure for 20–22 hr at 110°. Exps. 1 and 2 were performed with different preparations of hEGF. The calculated number of residues was based on five glutamic acid residues per mole of polypeptide.

* Tryptophan/tyrosine ratio was measured spectrophotometrically (17). Tr, trace.

obtained. All of the subsequent data were obtained with the pooled fractions of peak II, which contained approximately 200–300 μg of hEGF. The absolute amount of protein present in such small quantities is difficult to ascertain. Amino-acid analysis of an aliquot, containing approximately 80 μg of mouse EGF equivalents (binding competition assay), indicated the presence of 43 μg of protein, assuming complete recovery. It is not known whether any carbohydrate is present. In the biological experiments described below, the quantity of hEGF used was determined by the binding assay.

Chemical Properties of hEGF. The purity of the final preparation was examined by polyacrylamide disc gel electrophoresis under acid and alkaline conditions (Fig. 3). It may be seen that in each instance, hEGF migrates as a single band. Human EGF and mouse EGF migrate at approximately the same rate at pH 2.3. However, under alkaline conditions, hEGF migrates much more rapidly, suggesting that the net charge of hEGF at pH 9.5 is more negative than its mouse counterpart.

To establish whether the competitive binding activity of hEGF was associated with the stained band observed in the gel, we performed the following experiment. The alkaline gel

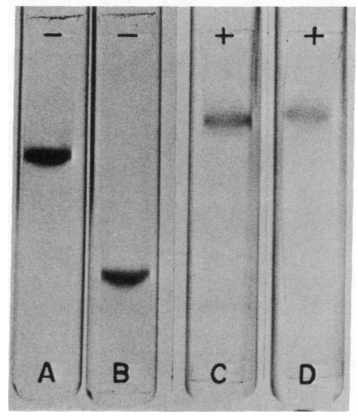

FIG. 3. Disc gel electrophoresis of hEGF and mEGF. Tubes A and C contain mEGF; tubes B and D contain hEGF. The pH of the gels in tubes A and B was 9.5 and in tubes C and D, 2.3. Samples of 10–20 μg of protein were applied, by methodology previously described (18).

(gel B, Fig. 3) was sliced into 1-mm sections, and each segment was fragmented in 400 μl of 10% $NaHCO_3$ containing 0.1% albumin and incubated overnight at 5°. The extract from each slice was assayed by competition with ^{125}I-labeled mEGF for binding to fibroblasts. Competitive binding activity was associated only with those fractions corresponding to the stained area of the gel.

The results of amino-acid analyses of 20- to 43-μg samples of hEGF are shown in Table 1, together with a comparison with mEGF. In view of the very small quantities of hEGF available for analysis, these data must be considered as preliminary. It is clear, however, that the two molecules exhibit both differences and similarities with respect to their amino-acid compositions. The minimal molecular weight of hEGF was estimated to be approximately 5500, assuming five glutamic acid residues per mole of protein.

The molecular weight of hEGF, as estimated by gel filtration, was approximately 5700. Gel filtrations were carried out on a column of Bio-Gel P-10, with cytochrome *c*, pancreatic trypsin inhibitor, and bacitracin as standard molecular weight markers in a solvent consisting of 0.1 M ammonium acetate. The elution volume of hEGF on the calibrated column was determined by the competitive binding assay.

The sedimentation equilibrium of hEGF was examined by Dr. Leslie Holladay at Vanderbilt University. Linear plots of ln concentration against r^2 (Fig. 4) revealed no significant heterogeneity, and a weight average molecular weight of 5291 was calculated. The partial specific volume of hEGF was calculated from the amino-acid composition (14) and found to be 0.710 cm^3/g.

Biological Properties of hEGF. The biological effects of mEGF include (*i*) stimulation of the growth of human foreskin fibroblasts, (*ii*) hypertrophy and hyperplasia of corneal epithelial cells in organ cultures, and (*iii*) induction of precocious eyelid opening when injected into newborn mice. All three effects have been duplicated, at least qualitatively, with pure hEGF.

The stimulation of thymidine incorporation into fibroblasts by nanomolar concentrations of hEGF is shown in Fig. 5. The effect of a saturating quantity of hEGF on the

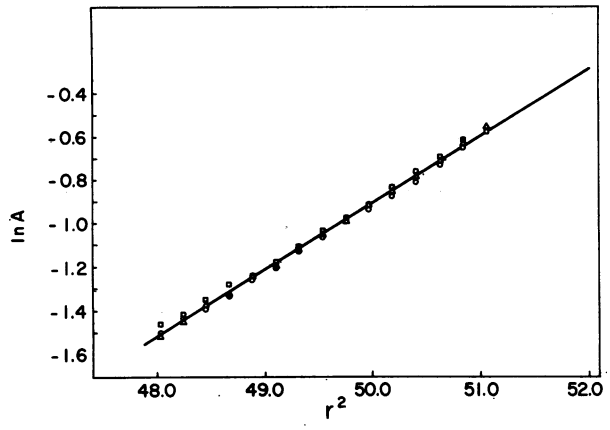

FIG. 4. Sedimentation equilibrium plot of ln A against the square of the radial distance (r) for hEGF. Determinations were made at 20° with a rotor velocity of 30,000 rpm for 31 hr before samples were scanned at 280 nm. The three sets of symbols represent three separate scans. The least squares line is shown.

growth of fibroblasts, as determined by cell number, in the presence of 1% and 10% calf serum, is presented in Fig. 6. At both serum concentrations, hEGF significantly increased the final cell densities reached.

Mouse EGF and hEGF, when assayed with organ cultures of the chick embryo cornea (15), were equally effective in causing the proliferation of the corneal epithelium. Histological results (not shown) identical to those previously described for mEGF (4) and recently described for hEGF (8),

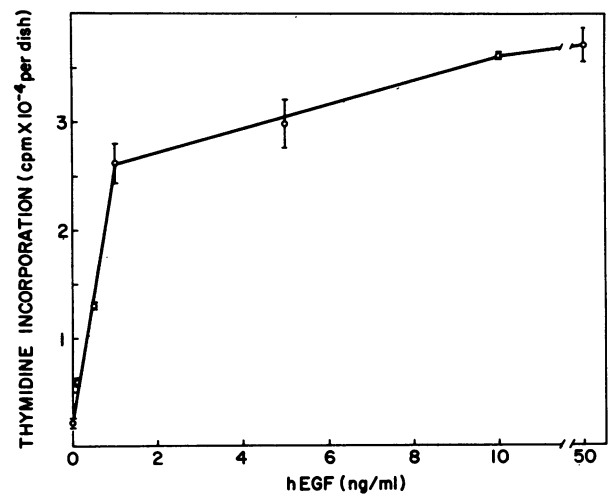

FIG. 5. Effect of hEGF concentration on the stimulation of [³H]thymidine incorporation. Human fibroblasts were plated in 60 mm culture dishes containing 5 ml of Dulbecco's medium plus 10% calf serum. When the cells became confluent, the medium was removed and the cell monolayers were washed twice with Hank's balanced salt solution. Five ml of Dulbecco's medium plus 1% calf serum were added to each dish and the cells were incubated for 48 hr. Indicated amounts of hEGF were then added to duplicate dishes, and 20 hr later, 1 μCi/ml of [*methyl*-³H]-thymidine (6.7 Ci/nmol) was added. The cells were labeled for 4 hr, and the radioactivity in the cold trichloroacetic acid-insoluble material was counted. Dishes contained approximately equal quantities of cellular protein (±10%). Cells stimulated by the addition of fetal calf serum (10% v/v) contained 3.9 × 10⁴ cpm per dish.

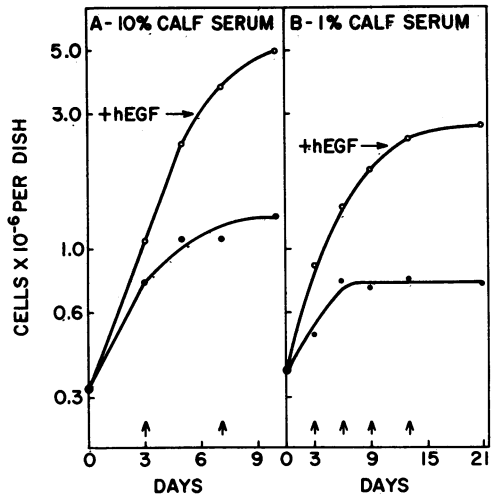

FIG. 6. Effect of hEGF on the growth of human foreskin fibroblasts. Fibroblasts were plated in 60 mm culture dishes containing Dulbecco's modified medium supplemented with (A) 10% calf serum, ●; 10% calf serum and hEGF (12.5 ng/ml), ○; (B) 1% calf serum, ●; 1% calf serum and hEGF (12.5 ng/ml), ○. Total cell counts in duplicate dishes were determined with a Coulter Counter and did not differ by more than 10%. Fresh medium, including hEGF, was added as indicated by arrows on the abscissa in the figure.

purified partially by affinity chromatography, were observed. The biological effects of both mouse and human EGF on the corneal epithelium were completely and specifically inhibited by the addition of excess quantities of the gamma globulin fraction prepared from a rabbit antiserum against mEGF.

Finally, the human EGF was assayed for precocious eyelid-opening activity by the daily subcutaneous injection into a newborn mouse (16). Control mice opened their eyes at 14 days; mEGF (1 μg/g per day or 0.25 μg/g per day) resulted in eyelid opening on day 9 or 11, respectively; hEGF (1 μg/g per day) resulted in eyelid opening on day 11. Human EGF thus appeared to be somewhat less active than mouse EGF in this *in vivo* mouse assay.

DISCUSSION

Human EGF was isolated by utilizing its ability to compete with ^{125}I-labeled mouse EGF in binding to human foreskin fibroblasts in culture. Ten grams of the starting material (an acetone powder obtained from approximately 15 liters of urine from pregnant women) contained approximately 700 μg equivalents of hEGF. Approximately 100–150 μg of pure hEGF were isolated by the procedures outlined in this paper. Although we have made use of urine from pregnant women, hEGF is not unique to pregnancy in that it is also present in urine from adult males.

The biological effects of hEGF, at least qualitatively, are similar to those previously described for mouse EGF. These include the stimulation of the growth of cultured human foreskin fibroblasts and corneal epithelial cells of the chick embryo in organ culture, and the *in vivo* induction of precocious eyelid opening in newborn mice.

Although hEGF appears to be biologically similar to mEGF, the physical and chemical properties of the two molecules are not identical. Human EGF appears to have a slightly lower molecular weight and a greater net negative

charge at an alkaline pH than the mouse EGF. The amino-acid compositions of the two polypeptides indicate certain similarities, such as the absence of phenylalanine and the presence of one methionyl and six half-cystinyl residues per mole.

The radioreceptor assay proved far more sensitive than a radioimmune assay based on crossreaction with antibodies to the mouse polypeptide (8), suggesting a closer relationship between the receptor binding sites than between the antigenic sites on the human and mouse polypeptides.

There is at present no direct evidence for the role of these growth factors in normal development and cell control. However, the presence of EGF-like molecules in both man and mouse suggests that an important function does exist.

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