Radioimmunoassay of Human Epidermal Growth Factor—hEGF Levels in Human Body Fluids

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By immunizing rabbits with purified human epidermal growth factor (hEGF), which was synthesized by *Escherichia coli* constructed by a genetic engineering technique, high titer antiserum for hEGF was obtained. The antiserum has an excellent specificity to hEGFs {hEGF, hEGF [1–51], hEGF [1–47] and [Leu²¹] hEGF} and has almost no cross-reactivity with other biological peptides, such as mouse EGF and human platelet derived growth factor. Using radioiodinated hEGF, a radioimmunoassay (RIA) for hEGF was established by a double antibody-polyethylene glycol method. As little as 10 pg/tube of hEGF was successfully determined by the present RIA methods. The intra- and inter-assay coefficients of variation for hEGF in human plasma or serum were 2.4–4.9% and 3.7–6.2%, respectively. The hEGF levels in human body fluids determined by the present method were as follows: urine (11–100 ng/ml), serum (1.0–1.8 ng/ml), plasma (0.2–0.8 ng/ml), sperm (17–47 ng/ml), saliva (0.9–3.0 ng/ml) and tears (9.5–27 ng/ml).

Keywords — radioimmunoassay; human epidermal growth factor (urogastrone); *Escherichia coli*; genetic engineering; extracellular biological fluid

In 1975, Cohen and Carpenter¹⁾ first published a procedure for the purification of human epidermal growth factor (hEGF), which is a polypeptide hormone comprised of 53 amino acids and has a growth stimulating activity on a number of epidermal and epithelial cells, both *in vivo* and *in vitro*.^{2–4)} In order to evaluate the role of hEGF in physiological or pathological states, a simple assay method with a high sensitivity for hEGF is needed. The development of radioimmunoassay (RIA) and enzyme immunoassay (EIA) for hEGF using human urinary EGF as the antigen have been reported.^{5,6)} We describe a simple and sensitive RIA using hEGF synthesized in *Escherichia coli* (*E. coli*) by a genetic engineering.⁷⁾ The results of hEGF levels in human body fluids, as measured by this RIA method, are presented and a comparison with reported levels of hEGF are discussed.

Materials and Methods

Materials — hEGF and [Leu²¹]-hEGF were synthesized in *E. coli* which was constructed by a genetic engineering technique.⁷⁾ The synthesized polypeptides were isolated by high performance liquid chromatography (HPLC).

The hEGF [1–51] and hEGF [1–47] were by-products of hEGF. Mouse EGF (mEGF), mouse nerve growth factors 2.5s and 7s (mNGF), bovine fibroblast growth factor (bFGF), human platelet derived growth factor (hPDGF), and human endothelial cell growth supplement (hECGS) were purchased from Collaborative Res. Inc. (U.S.A.). Other peptides and proteins were purchased from Sigma (St. Louis, Mo., U.S.A.). Carrier-free Na¹²⁵I (1 mCi/10 μ l) was purchased from the Radiochemical Center, Amersham (England).

Radioiodination of hEGF — The hEGF was labeled with ¹²⁵I according to the method of Greenwood,⁸⁾ with some modifications. To 10 μ l of an aqueous solution, containing 1.0 μ g of hEGF in a glass centrifuge tube, were added 20 μ l of 0.5 M phosphate buffer, pH 7.2, and 10 μ l of Na¹²⁵I solution (1.0 mCi/10 μ l). Then, 10 μ l of 3 mg/ml chloramine-T were added, and the reaction was stopped by the addition of 10 μ l of 3 mg/ml sodium metabisulfite about 1 min later. To the reaction mixture were added 100 μ l of 0.5% BSA, which was immediately applied to a Sephadex G-25 column (1.0 $\times$ 48 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The first radioactive

fraction was confirmed to be radioiodinated hEGF by retention behavior with reversed phase HPLC on a TSK ODS 120T column. The specific activity was 200 $\mu\text{Ci}/\mu\text{g}$ (about 1200 $\mu\text{Ci}/\text{nmol}$).

Preparation of Antiserum — Five rabbits (domestic albino) were immunized with hEGF. Approximately 1 ml of the hEGF solution (200 $\mu\text{g}/\text{ml}$) was emulsified with 1 ml of complete Freund's adjuvant and injected intradermally at multisites into the rabbits. This immunization was repeated six times at one week intervals. Blood samples were collected from the ear vein at one week intervals after the third injection and the separated antiserum was stored at 4 °C with 0.1% NaN_3 .

Radioimmunoassay Procedure — The standard diluent was made of 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl, 0.5% BSA and 0.1% NaN_3 (RIA buffer). The incubation mixture consisted of 0.5 ml of standard diluent, 0.1 ml of standard hEGF solution or sample solution, 0.1 ml of diluted antiserum (1 : 100000) and 0.1 ml of ^{125}I -hEGF solution (about 10000 cpm), which was incubated at 4 °C for 24 h. Then 0.1 ml of normal rabbit serum (1 : 100), 0.1 ml of goat anti-rabbit IgG serum (1 : 20) and 0.1 ml of polyethylene glycol 6000 (PEG 6000, 15%) were added to the incubation mixture which was allowed to stand for 30 min at 4 °C. After centrifugation at $3000 \times g$ for 30 min, the radioactivity of the precipitate was counted in a well-type gamma counter (Aloka, Tokyo, Japan).

Measurement of Anti-hEGF Serum Titers — The titers were determined by radioimmunoassay using a double antibody-PEG technique according to the method of Vaitukaitis⁹⁾ with some modifications. For the determination of the antibody titers, 0.1 ml of 2-fold serial dilution of the antiserum was incubated at 4 °C for 24 h in a polystyrene tube with 0.1 ml of ^{125}I -hEGF (10000 cpm). The following procedure was same as the double antibody-polyethylene glycol procedure described in the RIA procedure. After centrifugation at $3000 \times g$ for 30 min, the radioactivity of the precipitate was counted in a well-type gamma counter. The antiserum titers were indicated with a dilution

factor that bound 50% of counts per minute of ^{125}I -hEGF (10000 cpm).

Analysis of Human Body Fluids — All human body fluids used in this experiment were collected from healthy volunteers (men: 24–38 years old) who had not taken drugs for at least 2 weeks. Plasma samples were prepared by centrifugation of whole blood containing heparin at $1500 \times g$ for 10 min. Whole blood without heparin was allowed to clot spontaneously for 20 min at room temperature. After centrifugation at $1500 \times g$ for 20 min, serum samples were obtained. Plasma and serum samples were subjected to RIA without dilution. Urine was diluted 10-fold with RIA buffer. Saliva, sperm and tear samples were directly subjected to RIA.

Cross-Reaction Study — Twenty eight peptides and proteins were used for the cross-reaction study. The amounts required to reduce the binding of ^{125}I -hEGF by half were calculated from inhibition curves. The percentage of cross-reaction was determined according to the method of Abraham.¹⁰⁾

Results and Discussion

This report describes an immunoassay procedure for hEGF in body fluids.

The final purification of the recombinant hEGF was achieved by HPLC. Amino acid analysis and amino terminal sequence analysis of this purified peptide verified that the obtained compound was entirely identical with the authentic hEGF.

The antiserum was obtained from rabbits by immunization with the recombinant hEGF and was used to develop the RIA system. The time course of antibody production in rabbit serum is shown in Fig. 1. The results indicate that all sera from 5 rabbits at 9 weeks after the first immunization, had high titers and could be used in RIA.

A typical RIA calibration curve with the antiserum from rabbit no. 2 is shown in Fig. 2. The minimal detectable amount of hEGF was about 10 pg/tube. In view of coprecipitation or non-specific adsorption of ^{125}I -hEGF, the present double antibody precipitation procedure with polyethylene glycol was superior to the double antibody or charcoal-dextran method. This fact

Fig. 1. Titer Development Following Injections of hEGF in Rabbits no. 1–5 as Measured by RIA

Antibody concentration in rabbit antisera as detected by RIA: rabbits no. (○), no. 2 (●), no. 3 (△), no. 4 (▲) and no. 5 (□).

Fig. 2. A Typical RIA Calibration Curve for hEGF

Radioimmunoassays were carried out with 1:100000 dilution of the antiserum from rabbit no. 2.

Means of triplicate determinations of % bound ($100 \times B/B_0$) are plotted against the amount of the unlabeled hEGF.

TABLE I. Recoveries of hEGF Added to Human Serum and Urine^{a)}

	Added (std.)	hEGF (pg/tube)		Recovery (%)	N
		Found	Expected		
Serum	0	105			
	25	129	130	99.2	6
	50	155	155	100	6
	100	210	205	102.4	6
			Mean	100.5 ± 1.7	
Urine ^{a)}	0	88			
	25	110	113	97.3	6
	50	133	138	96.4	6
	100	187	188	99.5	6
			Mean	97.7 ± 1.6	

^{a)} Radioimmunoassays were carried out with a 1:100000 dilution of the antiserum from rabbit no. 2.

^{b)} Urine was used at a 20-fold dilution.

TABLE II. Precision of the Radioimmunoassay^{a)}

Sample	Intra-assay (n = 10)		Inter-assay (n = 6)	
	hEGF (ng/ml)	CV (%)	hEGF (ng/ml)	CV (%)
A (plasma)	20.7	4.8	20.0	6.2
B (plasma)	61.9	4.9	58.7	4.6
C (plasma)	79.3	2.4	80.6	3.7
D (serum)	169	3.3	168	5.1

^{a)} Radioimmunoassays were carried out with a 1:100000 dilution of the antiserum from rabbit no. 2.

CV = coefficients of variation.

TABLE III. Cross-Reactivities of the Anti hEGF Antiserum with Various Peptides and Proteins^{a)}

Various peptides and proteins	Cross reactivity (%)	Various peptides and proteins	Cross reactivity (%)
hEGF	100	Lys-Bradykinin	<0.01
hEGF[1–51]	97.5	CCK-Tetrapeptide	<0.01
hEGF[1–47]	96.0	α -Endorphin	<0.01
[Leu ²¹]-hEGF	99.5	Leu-Enkephalin	<0.01
Urinary hEGF	94.9	LH-RH	<0.01
mEGF	<0.01	Pepstatin	<0.01
mNGF	<0.01	Schizophrenia Related Peptide	<0.01
bFGF	<0.01	TRH	<0.01
hPDGF	<0.01	Cyt-c	<0.01
hECGS	<0.01	Ferritin	<0.01
Insulin	<0.01	β -Galactosidase	<0.01
hCG	<0.01	β -Lactamase	<0.01
Secretin	<0.01	Myoglobin	<0.01
Angiotensin I	<0.01	Trypsin inhibitor	<0.01
Angiotensin II	<0.01		

a) Radioimmunoassays were carried out with 1:100000 dilution of antiserum from rabbit no. 2.

TABLE IV. Concentrations of hEGF in Human Body Fluids^{a)}

Sample	hEGF (ng/ml) (<i>n</i> = 8)	Reported value (ng/ml) ¹⁶⁾
Urine	11–100	25–250
Plasma	0.2–0.8	0.1–0.2
Serum	1.0–1.8	0.5–1.5
Saliva	0.9–3.0	0.2–2.0
Sperm	17–47	10–100
Tear	9.5–27	ND ^{b)}

a) Radioimmunoassays were carried out with a 1:100000 dilution of the antiserum from rabbit no. 2.

b) not determined.

was confirmed with the experiment using ¹²⁵I-hEGF (10000 cpm), and the nonspecific binding was calculated as *ca.* 3% for the former procedure and *ca.* 5–10% for the latter procedure.

The dose-response curve was constructed with the antiserum from rabbit no. 2 which was diluted 1 : 100000. The ratio of the bound to free (B/F) observed with the antiserum was plotted against the concentration of antigen according to the method of Scatchard.¹¹⁾ The affinity constant of antiserum from rabbit no. 2 was thus estimated to be $1.1 \times 10^9 \text{ M}^{-1}$.

The recoveries of spiked hEGF in human serum or urine were quantitative and there was

no inhibition for the determination of spiked hEGF in human serum and urine (Table I).

Intra- and inter-assays coefficients of variation were 2.4–4.9% (*n* = 10) and 3.7–6.2% (*n* = 6) for plasma or serum samples, respectively (Table II). Thus, the present RIA proved to be satisfactory in both accuracy and precision.

The specificity of the antiserum was assessed by measuring the binding ability of various peptides and proteins to the antibody. The results of the cross reactivity are shown in Fig. 3 and summarized in Table III. It was also shown that the antiserum had almost no affinity to mEGF, mNGF, bFGF, hPDGF, hECGS and other various proteins and peptides, while hEGF[1–51],

Fig. 3. Cross Reactivity of Several Related Compounds

RIA using hEGF as the standard (○), and other related compounds: [Leu²¹]-hEGF (●) hEGF[1-51] (△), hEGF[1-47] (▲), urinary hEGF (□) and m-EGF (■). Means of triplicate determinations of % bound ($100 \times B/B_0$) are plotted against the amount of unlabeled peptides. Radioimmunoassays were carried out with a 1:100000 dilution of the antiserum from rabbit no. 2.

hEGF[1-47], [Leu²¹]-hEGF and urinary hEGF had the same affinity for the antiserum as hEGF. Additionally, almost no cross-reactivity of the anti hEGF antiserum to other components, including culture medium or bacterial components, indicated that the hEGF used was immunologically pure.

Since the antiserum against hEGF used in this report was prepared by using the hEGF synthesized in *E. coli* and not prepared from urine, it is assumed that the hEGF level in human fluids and tissues could be accurately determined with this RIA without the influences of other compounds in fluids and tissues.

As shown in Table IV, higher levels of hEGF were found in urine or sperm, compared to plasma, serum or saliva, confirming the trends reported by Hirata, *et al.*^{12,13} The significant difference between serum and plasma seems to be caused by the release of hEGF-like immunoreactivity from platelets during the blood coagulations.^{14,15} In the case of tears, high levels of hEGF were observed (9.5–27 ng/ml). This fact is interesting because tears are one of exocrinical body fluids and the possible role of hEGF in tears could be a protection of the cornea.

In view of simplicity, sensitivity and repro-

ducibility, the RIA system presented here was shown to be a useful method to evaluate the roles of hEGF-immunoreactive materials in human body fluids and tissues in physiological and pathological states.

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