Human Plasma Epidermal Growth Factor/β-Urogastrone Is Associated with Blood Platelets

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ABSTRACT Human epidermal growth factor (hEGF) has previously been isolated from urine and probably is identical to human β-urogastrone (hUG). Immunoreactive hEGF/UG has been found in the plasma of normal subjects. In this study, using immunoaffinity chromatography to extract hEGF/UG from plasma, we found that immunoreactive hEGF/UG in blood was associated with blood platelets. It was present in platelet-rich, but not platelet-poor plasma and serum, and was found predominantly in the platelet fraction of whole blood. Sephadex G-50 Fine gel-exclusion chromatography of an extract of outdated blood bank platelets revealed two hEGF/UG components, one of which eluted in the void volume, and the other of which coeluted with purified standard hEGF/UG. The former hEGF/UG component was a high-molecular weight form that was cleaved into hEGF/UG by incubation with either mouse EGF/UG-associated arginine esterase or trypsin. It appeared to be identical to the high-molecular weight hEGF/UG previously reported in human urine, except for its apparently equal activities in radioimmunoassay and radioreceptor assay. The latter hEGF/UG component was immunologically, biologically, and physiochemically indistinguishable from highly purified hEGF/UG from human urine and was immunologically different from purified human platelet-derived growth factor. Platelet-associated hEGF/UG may account for the mitogenic activity of serum in cell lines in which platelet-derived growth factor is not active. Since hEGF/UG appears to be liberated from platelets during coagulation, platelet-associated EGF/UG may be involved in normal vascular and tissue repair and in the pathogenesis of atherosclerotic lesions. The discovery that the EGF/UG in plasma is associated with blood platelets raises important new possibilities for its role in human health and disease.

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INTRODUCTION

Epidermal growth factor (EGF),[1] which was first isolated from male mouse submandibular glands, stimulates growth of many tissues (1). Human EGF (hEGF) was subsequently isolated from human urine (2, 3) and is probably identical to human β-urogastrone (hUG), a potent inhibitor of stimulated gastric acid secretion (4, 5). Immunoreactive EGF/UG concentrations in plasma and a variety of human tissues have previously been reported (6–10). The primary tissue source of the hEGF/UG found in plasma and urine, however, is still unknown.

Culture of most cells in vitro is known to require the presence of serum. It was postulated that a mitogenic factor or factors not present in plasma appeared in serum during its preparation from blood (11). Subsequently, considerable effort has been expended to identify the factors in serum that stimulate cell growth. One of these appears to be EGF/UG. Another potent mitogenic factor in serum is derived from blood platelets (12, 13). Human platelet-derived growth factor has recently been purified and partially characterized (14–17).

The present study was conducted to determine whether plasma immunoreactive hEGF/UG is associated with and perhaps derived from platelets or other formed elements in blood.

METHODS

Samples

Blood samples were obtained from healthy volunteer subjects after obtaining their informed consent. The subjects had taken no medications during the preceding 2 wk. 9 vol of peripheral venous blood were aspirated through 19-gauge

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; hEGF and hUG, human EGF and UG, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UG, urogastrone.

needles into syringes containing 1 vol of sterile acid/citrate/dextrose buffer, prepared according to National Institutes of Health Formula A: 8 g of citric acid monohydrate, 22 g of dextrose (anhydrous), and 26 g of sodium citrate (dihydrate) made up to 1 liter with distilled water.

In some experiments, units of clinically outdated human platelets (4–6 d old) were obtained from the American Red Cross Blood Bank.

Preparation of platelet-rich plasma, platelet-poor plasma, platelet-rich plasma-derived serum, platelet-poor plasma-derived serum, and platelet lysate

To prepare platelet-rich plasma, fresh whole blood samples were centrifuged at 180 *g* for 15 min at 20°C. The supernatant plasma constituted platelet-rich plasma. To prepare platelet-poor plasma, platelet-rich plasma was centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant fraction was aspirated and constituted the platelet-poor plasma. To prepare serum from platelet-rich plasma and platelet-poor plasma, sufficient 1 M $CaCl_2$ was added to each to yield a final calcium concentration of 20 mM. These calcium-enriched plasma preparations were incubated at 37°C for 2 h and then centrifuged at 10,000 *g* for 30 min at 4°C in order to prepare serum, which was aspirated from the pelleted plasma clot. Each of the plasma and serum preparations was frozen and thawed six times to rupture the platelets and was then centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant solutions were aspirated and stored frozen at −56°C before assay.

Outdated blood-bank platelets were concentrated by centrifugation at 3,200 *g* for 20 min at 4°C and washed twice with calcium- and magnesium-free phosphate-buffered saline solution (0.5 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 137 mM NaCl, 4 mM KCl, 11 mM glucose). The washed platelets were again concentrated by centrifugation, suspended in 2.5 ml of calcium- and magnesium-free phosphate-buffered saline containing 0.4 mM α-toluenesulfonylfluoride (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, NY), 0.1 mM leupeptin (Peptide Institute, Osaka, Japan), 10 μg/ml pepstatin (Peptide Institute), and 1,000 kallikrein inactivator units (KIU)/ml Trasylol (Aprotinin, Mobay Chemical Corp., Union, NJ). The platelet suspension was frozen and thawed six times to rupture the platelets and was then centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant solution, which constituted the platelet lysate, was aspirated.

Preparation of blood fractions

2 ml of freshly drawn acid/citrate/dextrose blood was diluted with 2 ml of Dulbecco's phosphate-buffered saline, carefully layered on 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) in a 10-ml polypropylene tube and centrifuged at 350 *g* for 40 min at 18°C. The samples were thereby separated into four layers (upper, interface, Ficoll, and erythrocyte layers) (18). The upper layer was aspirated and constituted the plasma preparation. The interface layer was transferred to a clean polypropylene tube, suspended in 4 ml of phosphate-buffered saline and centrifuged at 65 *g* for 10 min at 18°C. The pellet was suspended in 1 ml of the plasma fraction and constituted the lymphocyte preparation. The supernate was removed and concentrated by centrifugation, and the resulting pellet was resuspended in 1 ml of the plasma fraction and constituted the platelet preparation. The Ficoll layer was discarded. The erythrocyte layer was mixed with 1 ml of the plasma fraction and 0.4 ml of 4.5% Dextran T 500 (Pharmacia Fine Chemicals), transferred to a 12 × 75-mm polypropylene tube and allowed to settle at 4°C for 40 min. The supernate constituted the granulocyte preparation. Each of the preparations was frozen and thawed six times before subsequent immunoaffinity chromatography. The number of leukocytes and platelets in each preparation was counted manually in a hemocytometer (19) and was expressed as the percentage of the total number recovered after fractionation.

Assay procedures

Radioimmunoassay. Homologous hEGF/UG radioimmunoassay was performed as previously described (7, 8) with minor modifications. Two antisera were used, both raised in rabbits. In addition to the antiserum raised to highly purified hEGF/UG (3) (generously provided by Dr. S. Cohen, Vanderbilt University School of Medicine, Nashville, TN), whose specificity has previously been described (17), another antiserum generously provided by Dr. Y. Hirata, Kobe University School of Medicine, Kobe, Japan) with similar specificity, but greater sensitivity was also used. A stock buffer solution, consisting of 63 mM Na_2HPO_4, 13 mM Na_2EDTA, 0.02% (wt/vol) NaN_3, and 0.1% (vol/vol) Triton X-100, pH 7.4 (20), was prepared. The buffer was used as elution buffer for the Sephadex G-50 Fine (Pharmacia Fine Chemicals) gel-exclusion chromatography column that was used for purification of ^{125}I-labeled hEGF/UG. The radioimmunoassay diluent was prepared by adding 250 KIU/ml of Trasylol to the stock buffer and was used to dilute samples, standards, antisera, ^{125}I-labeled hEGF/UG, and second antibody. Extracts were assayed in duplicate at four or more threefold dilutions.

Highly purified hEGF/UG (3) (generously provided by Dr. S. Cohen) was used as both standard and labeled tracer. Human EGF/UG was labeled (21) with ^{125}I by dissolving 1 μg hEGF/UG in 10 μl 0.01 N HCl, adding 20 μl 0.5 M phosphate buffer, pH 7.4, containing 1 mCi ^{125}I ($Na^{125}I$, carrier-free, Amersham Corp., Arlington Heights, IL), and then 10 μl of a freshly prepared solution of chloramine-T, 2 mg/ml in 0.05 M phosphate buffer, pH 7.4. The reaction was allowed to proceed at 22°C for 15 s, after which it was stopped by adding 20 μl of a freshly prepared solution of sodium metabisulfite in 0.05 M phosphate buffer, pH 7.4 (22). The iodination mixture was immediately transferred to a 0.9 × 58-cm column of Sephadex G-50 Fine resin that had been equilibrated and was eluted at 22°C with the stock buffer solution; 1-ml fractions were collected. The fraction that coeluted with hEGF/UG and had the greatest number of counts per minute was diluted to ~1.5×10^7 cpm/ml, and 1-ml aliquots were stored frozen at −56°C. Specific activities of 350–400 μCi/μg were obtained. Labeled hEGF/UG was repurified immediately before each assay on the same Sephadex G-50 column, which had been washed exhaustively to reduce the radioactivity in the effluent to $<10^4$ cpm/ml.

A one-stage assay was performed, incubating for 3 d at 4°C. After incubation was complete, phase separation of antibody-bound from free peptide was achieved by adding 100 μl radioimmunoassay diluent containing 4 μl goat anti-rabbit immunoglobulin G serum (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), incubating the mixture for 3 h at 4°C, adding 1.6 ml ice-cold radioimmunoassay diluent containing 2.5% (wt/vol) bovine serum albumin (BSA), but not Triton X-100, and centrifuging for

20 min at 6,000 *g* at 4°C (20, 22). The supernates were decanted and the precipitates, which contained the antibody-bound hEGF/UG, were counted in a γ-scintillation spectrometer. The sensitivity (i.e., 15% displacement of ^{125}I-labeled tracer) is 3.5 pg hEGF/UG per tube. Intraassay and interassay coefficients of variation are 9.3 and 12.3%, respectively (n = 4).

The cross-reactivities of the following substances in the hEGF/UG radioimmunoassay were determined: arachidonic acid (99% pure, Sigma Chemical Co., St. Louis, MO), thromboxane B_2 (the gift of Dr. J. Pike, Upjohn Co., Kalamazoo, MI), platelet factor 4 (Abbott Laboratories, North Chicago, IL), β-thromboglobulin (Amersham Corp.), fibrinogen (Kabi, Stockholm, Sweden), thrombin (Parke Davis and Co., Detroit, MI), highly purified human platelet-derived growth factor (the gift of Dr. H. N. Antoniades, Institute for Blood Research, Boston, MA), mouse nerve growth factor (Collaborative Research, Inc., Waltham, MA), highly purified human β-lipotropin and γ-lipotropin (prepared in our laboratories), synthetic human β-lipotropin (35–56) and human adrenocorticotropin (Ciba-Geigy, Ltd., Basel, Switzerland), synthetic bovine γ_3-melanocyte-stimulating hormone (the gift of Dr. N. Ling, Salk Institute, San Diego, CA), synthetic human β-endorphin (the gift of Dr. J. Rivier, Salk Institute), purified human growth hormone and prolactin (National Hormone and Pituitary Program, Baltimore, MD), synthetic arginine vasopressin (Pierce Chemical Co., Rockford, IL), synthetic oxytocin and porcine pancreozymin (Sigma Chemical Co.), synthetic Met^5-enkephalin, somatostatin, and gonadotropin-releasing hormone (Beckman Instruments, Inc., Palo Alto, CA), synthetic thyrotropin-releasing hormone (Hoechst-Roussel Corp., Summerville, NJ), porcine insulin and glucagon (Eli Lilly & Co., Indianapolis, IN), porcine secretin (Warren-Teed, Columbus, OH), synthetic vasoactive intestinal polypeptide, substance P, eledoisin, bombesin, and neurotensin (Peninsula Laboratories, Inc., San Carlos, CA), synthetic pentagastrin (Ayerst Laboratories, New York), and human chorionic gonadotropin (Center for Population Research, National Institute of Child Health and Human Development).

β-Thromboglobulin was quantified using a commercial radioimmunoassay kit (Amersham Corp.).

Radioreceptor assay. In some experiments, EGF/UG radioreceptor assay using a human placental membrane fraction was performed as previously described (8), with minor modifications. The assay diluent was 100 mM Na_2HPO_4, 150 mM NaCl, 0.25% (wt/vol) BSA, pH 7.4. Mouse EGF/UG and hEGF/UG were found to compete indistinguishably with ^{125}I-labeled mouse EGF/UG for binding to receptor sites on the placental cell membranes. Therefore, the more abundant mouse EGF/UG, which was prepared in our laboratory by the method of Savage and Cohen (23), was used as ^{125}I-labeled tracer and standard.

Bioassay. [^{3}H]Thymidine incorporation by human foreskin fibroblasts was determined as previously described (24) as a measure of hEGF/UG biologic activity.

Immunoaffinity extraction

Extraction of hEGF/UG from blood samples was performed by immunoaffinity chromatography as previously described (10), but a batch method, rather than the column method, was used. An antiserum to highly purified hEGF/UG was raised in a rabbit and was found to have specificity similar to the antiserum previously described (10). When coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals), this antiserum had a binding capacity of 4.9 ng hEGF/UG per 100 μl of antibody-Sepharose suspension. Specimens were thawed and centrifuged at 6,000 *g* at 4°C for 20 min before extraction. A 1-ml aliquot of the specimen was mixed with 5 μl of 20% (vol/vol) Triton X-100, added to 200 μl of the antibody-Sepharose affinity gel suspension and incubated on an end-over-end rotator for 3 d at 4°C. The affinity gel was then washed twice with 1 ml of the radioimmunoassay diluent and once with 1 ml of 0.1 M ammonium acetate, pH 7.2. Adsorbed materials were eluted with 0.5 ml of 1 M acetic acid during 1 min of vigorous vortexing. The elution procedure was repeated three more times. The pooled eluates were lyophilized, stored frozen, and reconstituted in an appropriate diluent for subsequent analysis. In some experiments, this extraction procedure was scaled up fivefold and performed on outdated blood bank platelets. Extracts were pooled for subsequent analysis.

Recovery of hEGF/UG during the extraction procedure was estimated by adding a known amount of standard hEGF/UG to platelet-poor plasma and measuring the immunoreactive hEGF/UG in the eluates. There was no difference in the efficiency of extraction from plasma over the range of 50 to 500 pg/ml; it usually exceeded 90%.

Gel-exclusion chromatography

A 0.9 × 58-cm column was packed with Sephadex G-50 Fine resin, equilibrated, and developed at 4°C with 39 mM Na_2HPO_4, 11 mM KH_2PO_4, 77 mM NaCl, pH 7.4, containing 0.02% (wt/vol) NaN_3. A 1-ml aliquot of an immunoaffinity extract or enzyme incubation mixture was applied and eluted (11 ml/h, descending flow, 45 cm hydrostatic pressure); 1-ml fractions were collected. Recovery of ^{125}I-labeled hEGF/UG applied to the Sephadex G-50 column ranged from 86 to 98%.

In some experiments, a 1.6 × 90-cm column was packed with Sephacryl S-200 Superfine resin (Pharmacia Fine Chemicals), equilibrated and developed at 4°C with 0.1 M ammonium acetate, pH 7.2. A 2-ml aliquot of calibration standards or pooled void volume fractions from the Sephadex G-50 Fine column was applied and eluted (10 ml/h, descending flow, 55 cm hydrostatic pressure) and 2.5-ml fractions were collected. BSA (mol wt 69,000; Cohn fraction V, Sigma Chemical Co.), ovalbumin (mol wt 43,000; Worthington Biochemical Corp., Freehold, NJ), deoxyribonuclease I (mol wt 31,000; Worthington Biochemical Corp.), and ^{125}I-labeled hEGF/UG were used as calibration standards. Each fraction was subjected to hEGF/UG radioimmunoassay and radioreceptor assay, and fractions with hEGF/UG activity were pooled, lyophilized, and reconstituted in an appropriate diluent for subsequent analysis.

Digestion with mouse EGF/UG-associated arginine esterase or trypsin

High-molecular weight hEGF/UG was prepared by pooling immunoreactive hEGF/UG-containing eluate fractions from the Sephacryl S-200 Superfine column to which the lyophilized, reconstituted, pooled void volume eluate fractions from the Sephadex G-50 Fine column had been applied. 10 μg of high-molecular weight hEGF/UG was incubated either with 10 μg of mouse EGF/UG-associated arginine esterase (25, 26) (kindly provided by Drs. S. Cohen and J. M. Taylor, University of California, San Francisco) in 40 μl 100 mM Na_2HPO_4, 25 μM Na_2-EDTA buffer, pH 8.0, for 6 h at 37°C or with 0.1 μg trypsin (treated with L-1-to-

sylamide-2-phenylethyl chloromethyl ketone, 217 U/mg, Worthington Biochemical Corp.) in 100 μl 46 mM Tris-HCl buffer containing 1.15 mM $CaCl_2$, pH 8.1, at 37°C for 1 h. The trypsin reaction was stopped by adding 0.25 μg lima bean trypsin inhibitor (Worthington Biochemical Corp.). A control incubation was performed in the same buffer in the absence of either enzyme at 37°C for 6 h. Each incubation mixture was stored frozen until it was subjected to Sephadex G-50 Fine gel exclusion chromatography. Immunoreactive hEGF/UG concentrations were measured in the original high-molecular weight hEGF/UG preparation and each column eluate fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 6-mm (i.d.) tubes with 10-mm 3% acrylamide stacking gels and 100-mm 12.5% acrylamide separating gels (27). Human serum albumin (mol wt 66,000; Worthington Biochemical Corp.), ovalbumin, deoxyribonuclease I, and lysozyme (mol wt 14,300; Worthington Biochemical Corp.) were labeled with dansyl chloride and used as internal molecular weight markers (28). Each sample was heated in 1% SDS buffer containing 10 mM dithiothreitol in a boiling water bath for 2 min and then applied to the gel. After electrophoresis, the gel was cut into 1-mm slices, and the peptides in each gel slice were eluted by overnight incubation in 0.5 ml radioimmunoassay diluent at 37°C.

Isoelectric focusing

The isoelectric point (pI) was determined by isoelectric focusing (29) on 4% polyacrylamide disc gels containing carrier ampholytes (Ampholine, LKB Instruments, Inc., Rockville, MD), pH 2.5–6.0 and 2.5–4.8. After isoelectric focusing, gels were cut into 0.5-cm slices, and the hEGF/UG was eluted by overnight incubation in 500 μl of 10 mM NaCl at 37°C.

Statistical analysis

The unpaired Student's *t* test was used to compare mean values between corresponding groups.

RESULTS

Radioimmunoassay and radioreceptor assay of hEGF/UG. The specificity of the radioimmunoassay is summarized in Table I. No significant cross-reactivity was observed with large excesses of a variety of growth factors, hormones, and other substances. Thus, the radioimmunoassay measured only hEGF/UG and not human platelet-derived growth factor or other factors. Sensitivity for immunoreactive hEGF/UG was 15 pg/ml, using 100 μl of extract equivalent to 250 μl of plasma.

The competitive binding curves generated by platelet immunoaffinity extracts were parallel to those of hEGF/UG reference standard in both radioimmunoassay and radioreceptor assay (Fig. 1). The extracts had almost equal activity in both assays.

TABLE I
Specificity of hEGF/UG Radioimmunoassay

Materials	Quantity added per tube	Displacement of labeled hEGF/UG
	μg	
Human EGF/UG	0.00025	Complete
Fibrinogen, substance P, bombesin, eledoisin, Met^5-enkephalin, vasoactive intestinal polypeptide, neurotensin	100	0
Gastrin, somatostatin	25	0
Glucagon, oxytocin, adrenocorticotropic hormone	10	0
Thyrotropin-releasing hormone	5	0
Growth hormone, prolactin, arginine vasopressin, β-endorphin, gonadotropin-releasing hormone	1	0
Chorionic gonadotropin, β-lipotropin(35–56)	0.50	0
Platelet-derived growth factor, β-thromboglobulin, β-lipotropin, γ-lipotropin, bovine γ_3-melanocyte-stimulating hormone	0.25	0
Mouse EGF/UG	0.10	0
Platelet factor 4, arachidonic acid	0.01	0
Thromboxane B_2	0.005	0
Mouse nerve growth factor	0.001	0
Secretin, *25 U;* thrombin *10 U;* pork insulin, *10 U;* pancreozymin, *1 U*	—	0

Immunoreactive hEGF/UG concentrations in plasma and serum. Immunoreactive hEGF/UG was found only in platelet-rich plasma and platelet-rich plasma-derived serum prepared from five different blood specimens; platelet-poor plasma and platelet-poor plasma-derived serum were without hEGF/UG immunoreactivity (Table II). There was little β-thromboglobulin in platelet-poor plasma and platelet-poor plasma-derived serum, indicating that there was relatively little platelet release product in these preparations. The concentrations of immunoreactive hEGF/UG in platelet-rich plasma and platelet-rich plasma-derived serum were identical ($P > 0.2$).

Immunoreactive hEGF/UG concentrations in blood fractions. Immunoreactive hEGF/UG was found mostly in the platelet fraction of five different blood specimens fractionated by Ficoll-Paque density centrifugation; lesser concentrations were found in the lymphocyte fraction, and barely detectable levels in the granulocyte fraction and plasma (Table III). The concentration in the platelet fraction was significantly greater than that in the lymphocyte fraction (*P*

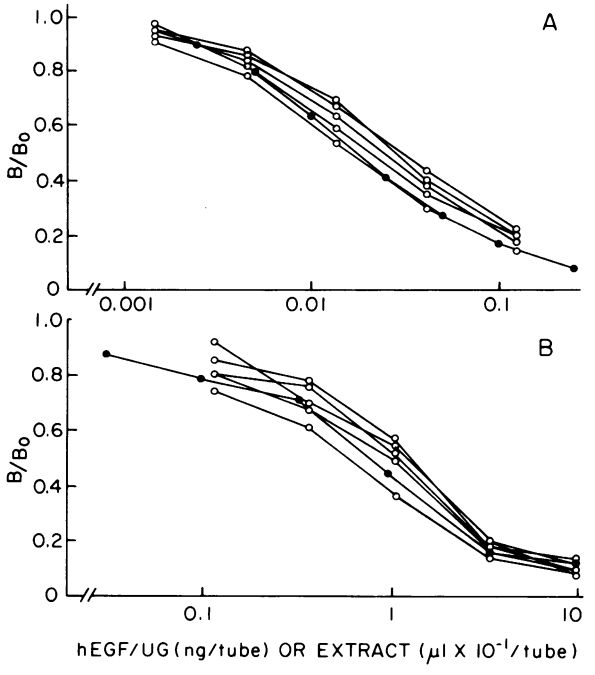

FIGURE 1 Competitive binding by platelet extracts in radioimmunoassay (*A*) and radioreceptor assay (*B*). Curves generated by highly purified hEGF/UG standard in radioimmunoassay and by highly purified mouse EGF/UG standard in radioreceptor assay (●); curves generated by immunoaffinity extracts of platelet lysates from five different units of outdated blood bank platelets (○).

< 0.005). The lymphocyte and platelet fractions were both prepared from the Ficoll-Paque interface layer, and the lymphocyte fraction was contaminated with platelets (Table III). The immunoreactive hEGF/UG concentrations in the lymphocyte and platelet fractions were more closely proportional to the platelet number in these fractions than to the leukocyte count (Table III). Thus, most, if not all, of the immunoreactive hEGF/UG in the lymphocyte fraction was probably on the basis of platelet contamination. Most of the immunoreactive hEGF/UG in the granulocyte fraction could be accounted for by the immunoreactive hEGF/UG in the plasma fraction in which the granulocytes were suspended.

Sephadex G-50 chromatography of platelet hEGF/UG. Pooled immunoaffinity extracts of outdated platelets were subjected to Sephadex G-50 Fine gel-exclusion chromatography, and the immunoreactive hEGF/UG and receptor-reactive hEGF/UG concentrations in the eluate fractions were determined (Fig. 2). Two hEGF/UG components were observed. One coeluted with purified standard low molecular weight hEGF/UG (K_d = 0.44; mol wt ~ 6,000) and, thus, appeared to be hEGF/UG. The other eluted in the void volume fractions (mol wt ≥ 30,000) and may have represented the high-molecular weight form of hEGF/UG previously isolated from human urine (8, 24). Both the low-molecular weight and high-molecular weight hEGF/UG components generated competitive binding curves parallel to that of standard hEGF/UG in the radioimmunoassay (Fig. 3) and radioreceptor assay (data not shown). However, both the low-molecular weight hEGF/UG and high-molecular weight hEGF/UG components had almost equal activity in both radioimmunoassay and radioreceptor assay, which was different from what had previously been reported (8).

Sephacryl S-200 chromatography and SDS-PAGE of platelet high-molecular weight hEGF/UG. A single peak of high-molecular weight hEGF/UG eluted from the Sephacryl S-200 Superfine column to which the pooled Sephadex G-50 Fine column void volume fractions had been applied (Fig. 4 *A*). It had almost equal activity in both radioimmunoassay and radioreceptor assay and an apparent molecular weight of ~42,000. Significant absorbance at 280 nm was observed in fractions of molecular weight ≥ 69,000, but these fractions had neither EGF/UG radioreceptor

TABLE II
Immunoreactive hEGF/UG and β-Thromboglobulin Concentrations in Plasma and Serum Prepared from Five Specimens of Blood

Fraction	Immunoreactive hEGF/UG						Immunoreactive β-thromboglobulin, mean±SEM
	No. 1	No. 2	No. 3	No. 4	No. 5	Mean±SEM	
				pg/ml			*μg/ml*
Platelet-rich plasma	220	140	364	251	391	273±46	14.3±2.6
Platelet-rich plasma-derived serum	109	136	338	322	463	274±66	15.0±1.8
Platelet-poor plasma	<15	<15	<15	<15	<15	<15	0.0270±0.0009
Platelet-poor plasma-derived serum	<15	<15	<15	<15	<15	<15	0.0265±0.0012

TABLE III

Immunoreactive hEGF/UG Concentrations and Platelet and Leukocyte Counts in Ficoll-Paque Fractions of Five Specimens of Blood

Fraction	Immunoreactive hEGF/UG						Platelets*	Leukocytes*
	No. 1	No. 2	No. 3	No. 4	No. 5	Mean±SEM	Mean±SEM	Mean±SEM
	pg/2 ml original blood						%	
Platelets	430	319	220	330	412	342±38	78.5±2.6	8.0±2.2
Lymphocytes	182	163	127	178	205	171±13	19.5±2.2	77.6±2.7
Granulocytes	30	<15	18	25	32	22±4‡	0.5±0.1	14.4±2.6
Plasma	<15	<15	20	27	<15	14±4‡	1.5±0.4	0

* Platelet and leukocyte counts in each fraction are expressed as the percentage of the total of platelets and leukocytes, respectively, recovered in all fractions combined.

‡ For purposes of calculating means, undetectable levels were assigned a value of 7.5 pg/2 ml original blood.

activity nor radioimmunoreactivity. Human EGF/UG has three internal disulfide bonds (4). Therefore, its molecular weight can accurately be estimated only under reducing, as well as denaturing conditions. When partially purified high-molecular weight hEGF/UG was subjected to SDS-PAGE in the presence of dithiothreitol, its apparent molecular weight was ~33,000 (Fig. 4 *B*), identical to that previously estimated for one of the urine high-molecular weight hEGF/UG moieties (8).

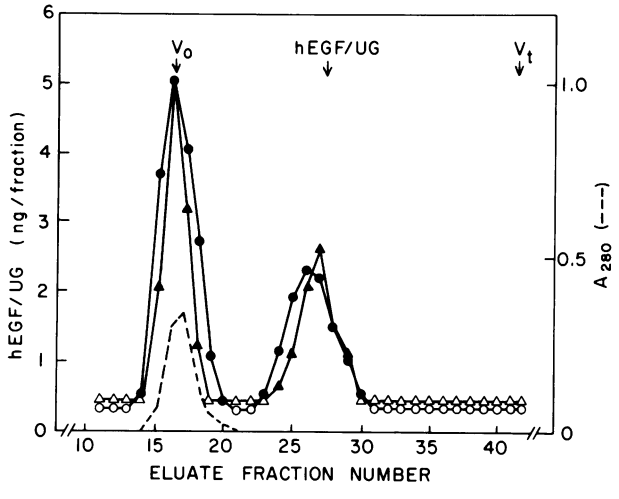

FIGURE 2 Sephadex G-50 Fine gel-exclusion chromatography of platelet extract. 1 ml of an immunoaffinity extract representing 3 units of outdated blood bank platelets was applied to a 0.9 × 58-cm column and eluted with phosphate-buffered saline; 1-ml fractions were collected. The elution positions of BSA (V_0, column void volume), ^{125}I-labeled hEGF/UG and NaCl (V_t, column total volume) are indicated. Immunoreactive hEGF/UG (●); radioreceptor reactive hEGF/UG (▲); absorbance at 280 nm (A_{280})(- - -). Open symbols indicate that hEGF/UG was undetectable at the concentration indicated.

Conversion of high-molecular weight hEGF/UG to low-molecular weight hEGF/UG by mouse EGF/UG-associated arginine esterase or trypsin. When partially purified high-molecular weight hEGF/UG from lysed platelets was incubated for 6 h without addition of enzymes, it still eluted from the Sephadex G-50 Fine column in the void volume fractions (Fig. 5 *A*). After incubation with either mouse EGF/UG-associated arginine esterase for 6 h (Fig. 5 *B*) or trypsin for 1 h (Fig. 5 *C*), two immunoreactive hEGF/UG components were observed as has previously been described for urinary high-molecular weight in hEGF/UG (24): high-molecular weight hEGF/UG and a low-molecular weight hEGF/UG that coeluted with purified standard hEGF/UG.

Biologic activity of platelet hEGF/UG. The biologic potencies of low-molecular weight and high-molecular weight hEGF/UG extracted from platelets was assessed by determining their effects on the incorpo-

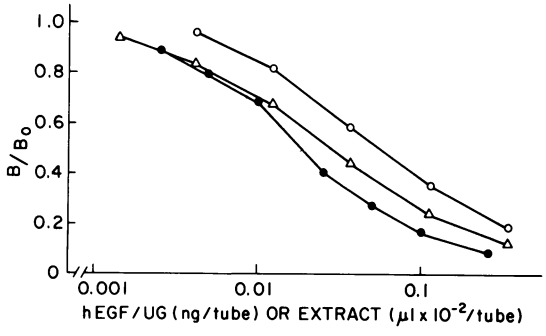

FIGURE 3 Competitive binding by platelet-associated low-molecular weight and high-molecular weight hEGF/UG in radioimmunoassay. Curves generated by highly purified hEGF/UG standard (●) and partially purified low-molecular weight hEGF/UG (○) and high-molecular weight hEGF/UG (△) prepared by Sephadex G-50 Fine gel-exclusion chromatography of platelet lysate.

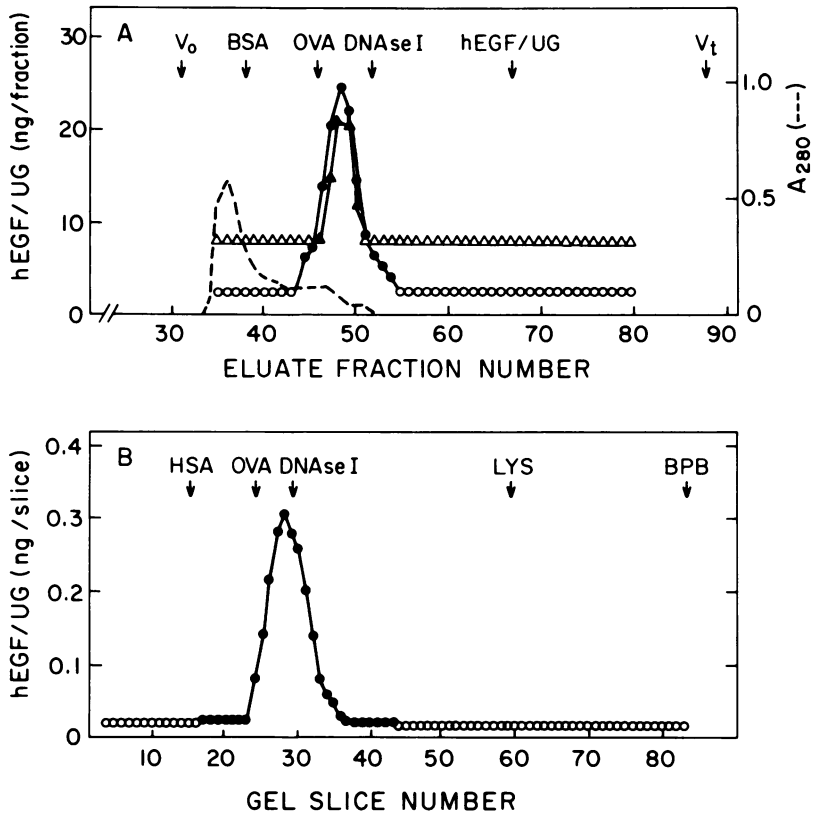

FIGURE 4 Sephacryl S-200 Superfine gel-exclusion chromatography and SDS-PAGE of platelet-associated high-molecular weight hEGF/UG. (*A*) Pooled void volume fractions from the Sephadex G-50 Fine column were applied to a 1.6 × 90-cm Sephacryl S-200 Superfine column and eluted with 0.1 M ammonium acetate, pH 7.5; 2.5-ml fractions were collected. The elution positions of calibration standards are indicated: BSA, bovine serum albumin (mol wt 69,000); OVA, ovalbumin, (mol wt 45,000); DNase I, deoxyribonuclease I (mol wt 31,000); and ^{125}I-labeled hEGF/UG. Data are plotted as in Fig. 2. (*B*) Pooled hEGF/UG-containing fractions from the Sephacryl S-200 Superfine gel column were lyophilized, incubated in the presence of 1% SDS and 10 mM dithiothreitol and applied to the 12.5% polyacrylamide gel. Elution positions of dansylated internal standards used to calibrate the gel are indicated: HSA, human serum albumin (mol wt 66,000); OVA, ovalbumin (mol wt 45,000); DNase I, deoxyribonuclease 1 (mol wt 31,000); LYS, lysozyme (mol wt 14,300); BPB, bromphenol blue. Data are plotted as in Fig. 2.

ration of [^{3}H]thymidine by human foreskin fibroblasts and were comparable to that of standard hEGF/UG (Fig. 6). Half-maximal stimulation was observed at concentrations of ~0.2 and 0.4 ng/ml of low-molecular weight and high-molecular weight hEGF/UG, respectively, based on hEGF/UG radioimmunoassay measurement of mass. At higher concentrations, platelet-derived low-molecular weight hEGF/UG had less effect on [^{3}H]thymidine uptake. This appeared to be due to contamination by inhibitory substances, since addition of low-molecular weight hEGF/UG at a concentration of 1.26 ng/ml to a maximally stimulating concentration (10 ng/ml) of standard hEGF/UG reduced uptake by 67% (data not shown).

Isoelectric focusing of platelet hEGF/UG. The low-molecular weight hEGF/UG material eluted from the Sephadex G-50 Fine gel column appeared to consist of two components, the more abundant of which appeared to have a pI of ~4.2, and the other of which appeared to have a pI of ~4.5 under the same conditions (Fig. 7). These materials both were about half as active in the radioreceptor assay as in the radioimmunoassay. The high-molecular weight hEGF/UG appeared to have a pI of ~3.8 (Fig. 8).

DISCUSSION

The homologous radioimmunoassay for hEGF/UG has made it possible to measure hEGF/UG concentrations

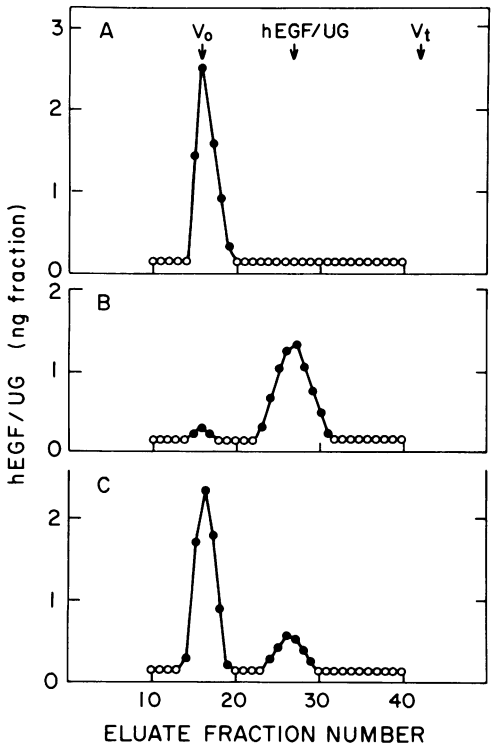

FIGURE 5 Sephadex G-50 Fine gel-exclusion chromatography of high-molecular weight hEGF/UG. Platelet-associated high-molecular weight hEGF/UG was incubated without enzymes (*A*), with mouse EGF/UG-associated arginine esterase (*B*), or with trypsin (*C*). Incubation mixtures were applied to a 0.9 × 58-cm Sephadex G-50 Fine column and eluted with phosphate buffer; 1-ml fractions were collected. Data are plotted as in Fig. 2.

in human fluids and tissue extracts (7, 10, 30). However, unextracted plasma failed to generate radioimmunoassay competitive binding curves parallel to that of standard hEGF/UG (6, 8). Anti-hEGF/UG immunoglobulin G-Sepharose affinity chromatography has previously been shown to be a useful and reliable method for extracting hEGF/UG from tissue homogenates and plasma and for excluding other proteins and substances that may interfere in the radioimmunoassay system (9, 10).

We found immunoreactive hEGF/UG only in platelet-rich plasma and serum preparations in which the platelets had been lysed by repeated freeze-thawing. The concentration in the serum was similar to that in the plasma from which it had been prepared by addition of calcium, indicating that the process of coagulation neither augmented nor diminished the amount of immunoreactive hEGF/UG released by freeze-thawing alone.

Using immunoaffinity chromatography to extract hEGF/UG from various Ficoll-Paque blood fractions,

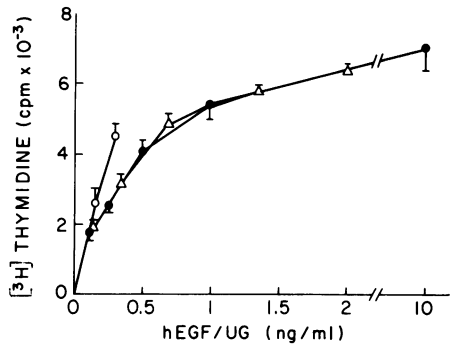

FIGURE 6 Stimulation of [^{3}H]thymidine incorporation into cultured human foreskin fibroblasts by hEGF/UG. Highly purified standard hEGF/UG (●) and platelet-associated low-molecular weight hEGF/UG (○) and high-molecular weight hEGF/UG (△) extracted from platelet lysates by immunoaffinity chromatography and separated by Sephadex G-50 Fine and Sephacryl S-200 Superfine gel-exclusion chromatography were added to confluent cells. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was measured. Each point indicates the mean of triplicate cultures; the bracket indicates the SEM. Values were corrected by subtracting trichloroacetic acid-insoluble [^{3}H]thymidine counts per minute incorporated into unstimulated control cultures.

we found that most of the hEGF/UG was associated with platelets. The lesser concentration of immunoreactive hEGF/UG in the lymphocyte fraction ap-

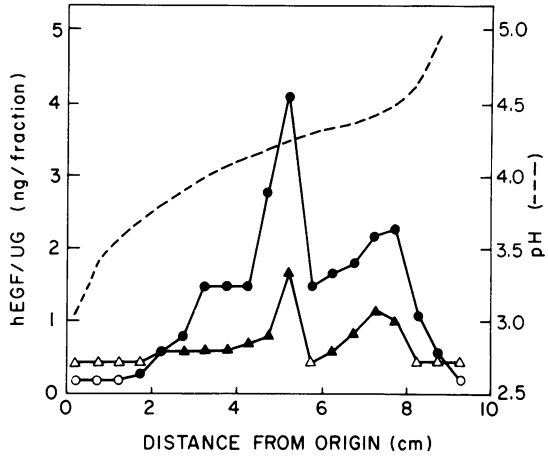

FIGURE 7 Isoelectric focusing of low-molecular weight hEGF/UG from immunoaffinity extracts of lysed platelets. Pooled low-molecular weight hEGF/UG-containing fractions from the Sephacryl S-200 Superfine gel-exclusion column were desalted, lyophilized, and applied to a polyacrylamide disc gel containing carrier ampholytes, pH 2.5–6.0. Immunoreactive hEGF/UG (●); radioreceptor reactive hEGF/UG (▲); pH gradient, as actually measured in the eluate from individual gel slices (- - -). Open symbols indicate that hEGF/UG was undetectable at the concentration indicated.

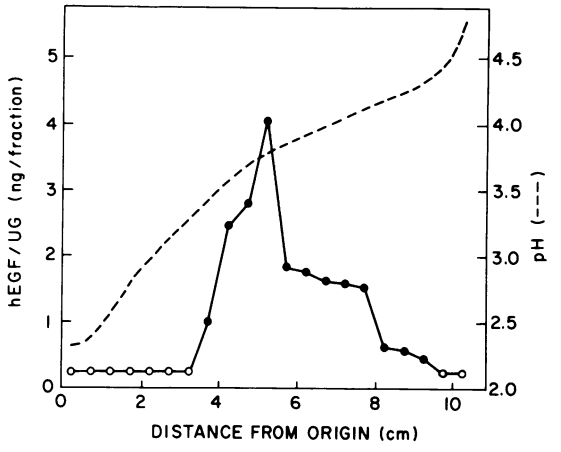

FIGURE 8 Isoelectric focusing of immunoreactive high-molecular weight hEGF/UG from immunoaffinity extracts of lysed platelets. Pooled high-molecular weight hEGF/UG-containing fractions from the Sephacryl S-200 Superfine gel-exclusion column were desalted, lyophilized, and applied to a polyacrylamide disc gel containing carrier ampholytes, pH 2.5–4.8. Data are plotted as in Fig. 7.

peared to be due to platelet contamination of that fraction.

The concentrations of immunoreactive hEGF/UG we found in platelet-rich plasma exceeded those previously reported in whole plasma (10). There are several potential explanations for this difference, among them the possibility that procedures for collecting blood and preparing plasma in which special care is not taken to recover platelets may result in loss of immunoreactive hEGF/UG from the resultant plasma. In addition, the increased affinity of anti-hEGF/UG immunoglobulin G-Sepharose used in the present study for high-molecular weight hEGF/UG may also help to explain not only the increased total immunoreactive hEGF/UG concentrations we measured, but also the greater ratio of high-molecular weight hEGF/UG to low-molecular weight hEGF/UG found in plasma in this, as compared to the previous study (10).

The serum concentrations of immunoreactive hEGF/UG we observed are still less than those required for half-maximal stimulation of fibroblast growth. Virtually none of this hEGF/UG exists free in plasma, however. Rather, it is associated with platelets. Thus, if it is liberated during platelet aggregation, as it seems to be, then local hEGF/UG concentrations, at the site of vascular injury, for example, may greatly exceed those we have observed in whole serum.

Platelet-associated low-molecular weight hEGF/UG appeared to be immunologically, biologically, and physiochemically indistinguishable from highly purified hEGF/UG standard, except for the existence of two forms detected by isoelectric focusing. βUG and γUG were reported to have pIs of 4.5 and 4.3, respectively, and to differ by deletion of a single COOH-terminal Arg residue from βUG (4). Thus, it is possible that the two components observed in this study with pIs of 4.5 and 4.2 represent hEGF/βUG and hEGF/γUG, respectively. Both appeared to have similar immunoreactivity/bioactivity ratios.

Platelet-associated high-molecular weight hEGF/UG appeared to be immunologically and physiochemically similar to urinary high-molecular weight hEGF/UG (8, 24). However, it differed from urinary high-molecular weight hEGF/UG in that its biologic activity appeared to be comparable to its immunological activity. Further characterization of high-molecular weight hEGF/UG from both sources is needed to elucidate their possible relationship to one another and to low-molecular weight hEGF/UG.

We observed significant concentrations of nonimmunoreactive nonradioreceptor reactive A_{280} material in the void volume fractions of the Sephadex G-50 Fine column to which the immunoaffinity extracts were applied (Fig. 2). Proteins adsorb nonspecifically to agarose gels, however, and can be eluted by the extremes of pH, chaotropic ions, or high salt concentrations that are used to dissociate antigen-antibody complexes on immunoaffinity gels. Such nonspecific adsorption can be minimized by first exposing the extract to agarose gel to which nonimmune serum has been covalently bound and then incubating the nonadsorbed extract with the immunoaffinity gel. However, since the aim of this study was to extract and concentrate as much of the hEGF/UG materials in the samples as possible, rather than attempt to purify them, the nonimmune absorption step was omitted. When the Sephadex G-50 void volume fractions were subsequently analyzed on the Sephacryl S-200 column, the immunoreactive hEGF/UG was clearly separated from most of the protein that had nonspecifically adsorbed to the affinity gel (Fig. 4).

Platelet lysates have been reported to contain two different types of growth factors, cationic platelet-derived growth factor (14–17) and anionic growth factor(s) (17, 31). Cationic platelet-derived growth factor has been purified and shown to exist in multiple forms (mol wt ~ 27,000–35,000) that are converted into biologically inactive subunits in the presence of reducing agents (14, 17). Cationic platelet-derived growth factor is stored in the α-granules of platelets and is released during the granule release reaction (32, 33). There has been less investigation into the nature of the anionic platelet-derived growth factor, which has been reported to consist of two components (mol wt ~ 10,000 and ~ 40,000) and to have a pI of ~5.0 (34). The results of the present study are consistent with the possibility that platelet-associated low-mo-

lecular weight hEGF/UG and high-molecular weight hEGF/UG are identical to the two components of anionic platelet-derived growth factor. The association of hEGF/UG with platelets may account for the mitogenic activity of platelet lysate in numerous cell lines, the growth of which is not stimulated by cationic human platelet-derived growth factor (35).

Since platelet-associated hEGF/UG is released into plasma during the process of coagulation (unpublished observations), it may be an important factor in the repair of tissue damage. Furthermore, since EGF/UG stimulates growth of vascular smooth muscle cells (36), a phenomenon that is thought to be important in the pathogenesis of atherosclerosis (37), platelet-associated hEGF/UG may be involved in the genesis of human vascular disease.

The source of the hEGF/UG associated with blood platelets is not yet clear. Platelets do not have protein biosynthetic capability. It is possible either that hEGF/UG is synthesized by megakaryocytes and stored in platelets or that hEGF/UG is secreted into the blood by some other tissue(s) and is by some mechanism taken up by platelets. In either case, the hEGF/UG is later liberated into plasma during the coagulation process. Whatever its source, the knowledge that hEGF/UG is associated with and liberated from platelets raises important new possibilities concerning its role in human health and disease.

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