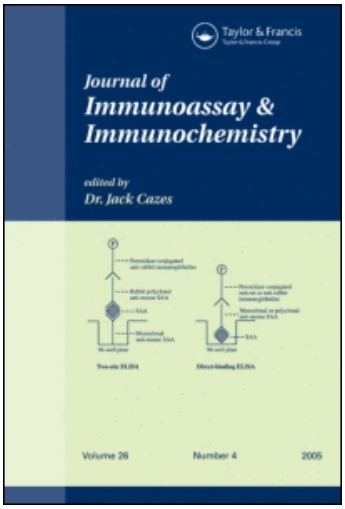

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Effect of Blood Collection Tube Types on the Measurement of Human Epidermal Growth Factor

Aysegul Yucel[a]; Resul Karakus[a]; Cemalettin Aybay[a]
[a] Faculty of Medicine, Department of Immunology, Gazi University, Ankara, Turkey

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Effect of Blood Collection Tube Types on the Measurement of Human Epidermal Growth Factor

Aysegul Yucel, Resul Karakus, and Cemalettin Aybay
Faculty of Medicine, Department of Immunology, Gazi University, Ankara, Turkey

Abstract: We observed significant differences in measured human epidermal growth factor (hEGF) levels for the same individual's serum/plasma samples between different tube types (glass, polystyrene, plastic with clot activator, plastic without clot activator, plastic with EDTA, polypropylene tubes). For all individuals, hEGF levels in plasma were found to be below the detection limit. The discrepancy of the hEGF levels in serum and plasma was attributed to the platelet derived EGF by analyzing platelet lyzate with size exclusion chromotography and demonstrating the immunoreactivity of the fractions corresponding to the pre-proEGF and/or proEGF elution time. Besides, samples of females showed much higher EGF levels than those of males in certain test tube types.

As a conclusion, all blood samples should be taken and stored in the same type of test tubes in order to make precise measurements for hEGF. And, the measured hEGF level in blood is susceptible to changes with blood clotting.

Keywords: EGF, ELISA, Serum, Plasma, Test tubes

INTRODUCTION

Epidermal growth factor (EGF) is an important cytokine that stimulates proliferation and differentiation of epidermal and epithelial cells in animals and acts as a potent mitogenic factor for many different types of cells *in vitro*.[1] However, the exact physiological role of EGF still remains to be elucidated.[2]

Address correspondence to Aysegul Yucel, M.D., Ph.D., Faculty of Medicine, Department of Immunology, Gazi University, 06510 Besevler, Ankara, Turkey. E-mail: aatakyucel@yahoo.com

EGF peptide is widely distributed in nearly all body fluids and secretions in varying concentrations.[3] Measurements of the levels of factors like EGF are useful for understanding pathogenesis and serve as prognostic and diagnostic indicators for many diseases including cancer. The use of appropriately collected and stored test samples and of sensitive and accurate methods have pivotal roles in these analyses.[4] The effect of sample collection, processing, and storage on the levels of cytokines measured by immunoassays in biological fluids has been shown in different studies.[4–6] During a study regarding human EGF (hEGF), we noticed that hEGF levels measured for the individual's same blood sample differed notably between different types of test tubes. Based on this observation, we investigated the effect of different blood collecting tubes on the measurement of EGF and any possible variations according to gender.

EXPERIMENTAL

Reagents

The following materials were purchased from commercial suppliers: recombinant human epidermal growth factor (rh-EGF) (Sigma, St. Louis, MO, USA), Bovine serum albumin (BSA-fatty acid free) (PAA Laboratories GmbH, Linz, Austria), EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL, USA), Streptavidin-horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA, USA), Ficoll (Lympho separation medium, ICN, Costa Mesa, CA, USA), Heparin-Sodium (Liquemin, 25,000 IU/5 mL iv, Roche Pharma-Schweiz, Reinach, Switzerland), 0.9% NaCl (Vacoliter, Isotonic NaCl solution, sterile, Eczacibasi/Baxter, Turkey).

Anti-hEGF Monoclonal Antibodies

Anti-hEGF monoclonal antibodies (mAb), purified from hybridoma clones 3E12a and 2B6a, were kindly provided by ImmunoGuide Laboratories (ImmunoGuide, Erdem Biotechnology Ltd, Cyberpark, Bilkent, Ankara, Turkey). 3E12a mAb (IgG1κ) was used as the capture antibody and 2B6a mAb (IgG1κ) was labelled with biotin according to manufacturer's instructions and used as the tracer antibody. Isotype determinations for both monoclonal antibodies were performed using a commercially available kit (ImmunoPure Monoclonal Antibody Isotyping Kit I [HRP/ABST], Pierce Biotechnology, Inc., Rockford, IL, USA).

Peripheral Venous Blood Samples

Human serum or plasma samples, and platelets were separated by centrifugation from peripheral venous blood collected from healthy volunteers

among laboratory personnel with a written informed consent. In the first set of experiments, 10 female and 10 male volunteers, in the second set of experiments, 17 female and 17 male volunteers, and in the third set of experiments, a male volunteer donated blood. The informed consent from all individuals included in this study was obtained. The peripheral venous blood samples (18–20 mL/person) were taken using disposable syringes with 21-gauge needle (Yu-Shou, Wuxi Yushou, Medical Appliances Co., Wuxicity, Jiangsu, China). Blood samples were immediately transferred into 6 different types of test tubes after taking off the needle, in order to prevent any risk of haemolysis and opening up the lid of test tube. No vacutainer system was used for blood collection.

Test Tubes and Storage

The test tubes used in the first set of experiments were as follows: 1-Sterile round bottom glass tube, 13 × 10 mm (Isolab Laborgerate GmbH, Germany); 2-Polystyrene (PS) sterile tissue culture tube (Greiner Labortechnik, Kremsmuenster, Austria, Catalog number: 164.160); 3-Plastic BD Vacutainer SST II Plus serum separation tube (with clot activating gel), (vac-no gel), 8.5 ml, 16 × 100 mm, (BD Vacutainer Systems Preanalytical Solutions, Belliver Industrial Estate, Plymouth, USA, Catalog number: 367953); 4-Plastic Vacuette serum separation tube Z-serum clot activator (without clot activating gel), (vac-no gel), 9 mL, (Greiner Bio-One Vacuette, Kremsmuenster, Austria, Catalog number: 455092/A06034); 5-Plastic sterile Vacuette K2E plasma separation tube with EDTA (K2EDTA), 10 mL, (Greiner Bio-One Vacuette, Kremsmuenster, Austria, Catalog number: 455038/0491A3). These tubes were chosen for plasma separation since EDTA was found to be an optimal anticoagulant for this purpose and other anti-coagulants were reported to alter the measured plasma levels for hormones or cytokines.[7] 6-Sterile polypropylene (PP) test tube with conical bottom and blue lid, 15 mL, (Greiner CellStar, Catalog number: 188.271, Greiner Labortechnik, Frickenhausen, Germany).

Blood samples in different tubes were kept at +4°C overnight and centrifuged at 1600 g (3K15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 15 min at +6°C and used for ELISA study.

Platelet Isolation

20 mL of peripheral venous blood sample was taken using 20 mL sterile disposable syringe from a male volunteer. The blood sample was immediately transferred into a 50 mL conical bottomed PP tube (CellStar, Greiner Labortechnik, Frickenhausen, Germany) containing 0.5 mL of heparin after taking off the needle. Blood and heparin were thoroughly mixed. 20 mL of 0.9%

NaCl solution was added to the heparinized blood and again thoroughly mixed. It was divided into two and each part was layered on top of 20 mL of Ficoll in a separate tube. Tubes were centrifuged at 400 g for 30 min. The platelet-rich plasma was collected as described elsewhere.[8] The collected fluid from both tubes were combined and 3 volumes of 0.9% NaCl was added and mixed thoroughly and then centrifuged at 1600 g for 10 min to wash off plasma constituents. The platelet pellet was washed once more with 0.9% NaCl. The resultant pellet was counted for platelets, both under light microscope, and by using an automated haemocytometer (Abbott-Cell-DYN-4000, Abbott Park, IL, U.S.A). The total number of platelets to be lyzed and used was about 75×10^7. In order to lyse the platelets, 2.5 mL of distilled water containing sodium-dodecyl-sulfate at 0.01% was added and vigorously vortexed and kept at room temperature (RT) for 30 min. By examining under a light microscope, all platelets were observed to be lyzed. A volume of 2.5 mL was passed through a 0.45 μm filter (FP 030/2, Schleicher and Schuell, Inc., Keene, NH, U.S.A) and 2 mL of the filtrate was loaded for size-exclusion chromatography.

Size Exclusion Chromatography

Pro-EGF and mature-EGF contents of the lyzed platelet sample were analyzed using a high performance liquid chromatography system (HPLC, 1100 Series, HPLC System, Agilent Technologies GmbH, Waldbronn, Germany) equipped with a 2 mL sample loop. A TSKgel G3000SW packed stainless steel column (21.5 mm × 300 mm, 13 μm particle size, TosoHaas, Japan) was attached to the HPLC system. The original chromatography software (ChemStation for LC 3D, Rev. A.08.03 Agilent Technologies 1999-2000) was used for data analysis. After equilibrating the column with PBS, pH 7.4, 2 mL of sample was loaded after filtering through 0.45 μm filter. The separation was performed at a constant flow rate of 1 mL/min at RT. Elution of protein was monitored by UV detector at 280 nm. Each fraction was collected in PP tubes at 1 min intervals and then the tubes were analysed for their EGF contents.

hEGF ELISA

High binding capacity ELISA plates (Costar, Corning Incorporated, Corning, NY, USA) were coated with 100 μL/well of capture mAb 3E12a at 1 μg/mL in 0.05 M carbonate-bicarbonate buffer pH 9.6 by incubating overnight at +4°C. Plates were washed 3 times with PBS plus 0.05% Tween 20 (PBS-T). 200 μL of phosphate buffered saline (PBS) containing 1% BSA (PBS-1% BSA) was added into each well and then incubated for 2 hrs at RT for blocking. Plates were washed 3 times with PBS-T. 100 μL of standards or

samples to be tested were added into corresponding wells. Standards were studied in duplicate and arithmetic means were used for calculations. Plates were then incubated for 1 hr at RT. After washing 3 times with PBS-T, 100 μL/well biotinylated tracer mAb 2B6a was added and plates were incubated for 1 hr at RT. After washing 3 times with PBS-T, 100 μL of streptavidin-horseradish peroxidase was added to each well and then incubated for 30 min at RT. After washing three times with PBS-T, the reaction was revealed with 100 μL/well 3,3′,5,5′ tetramethylbenzidine solution at RT. The reaction was stopped after 20 min with 100 μL of 1M H_2SO_4 per well and the optical density (OD) at 450/620 nm was measured with an ELISA reader (Sunrise Remote/Touch Screen, Tecan Austria GmbH, Grödig, Austria).

Statistical Analyses

Statistical analyses were done by Student's t-test. A Mann Whitney U-test was used for comparison of unrelated groups. Differences were considered statistically significant if $p < 0.05$. Calculations were performed using the SPSS for Windows Version 10.0 computer based statistics program.

RESULTS

hEGF Values in Blood Samples Taken into 6 Different Types of Test Tubes

Blood collected from 10 female (29.7 ± 6.7 years of age) and 10 male (33.3 ± 10.7 years of age) volunteers was transferred into 6 different types of test tubes as described above. The level of EGF measured was highest in polystyrene (PS) tubes, then followed by glass, plastic BD vacutainer SST II Plus with clot activator (vac-gel), plastic Greiner Vacuette without clot activator (vac-no gel), Greiner polypropylene (PP) tubes, and in plasma (plastic K2EDTA tubes), respectively. The EGF level in plasma separated from blood kept in K2EDTA containing plastic tubes was found to be below the detection limit in all dilutions, in all volunteers, both in females and males. The serum hEGF levels in 6 different test tubes are shown in Figure 1. We noted significant differences in hEGF levels when different types of blood collecting tubes were compared. However, there was no statistically significant difference between PS and glass tubes in this aspect ($p > 0.05$). Glass tubes significantly differed, in regard to hEGF content, from plastic vac-gel and plastic vac-no gel tubes ($p < 0.05$). Sera separated from blood kept in glass tubes also showed a much higher hEGF concentration than in PP tubes ($p < 0.05$). A very similar result was obtained for sera separated from blood kept in PS tubes when compared with vac-gel, vac-no

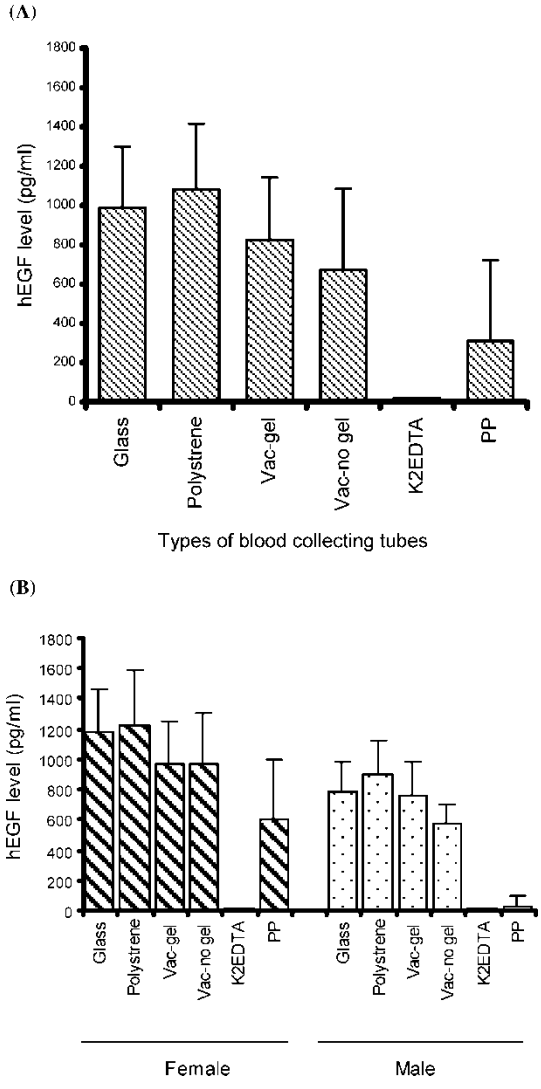

Figure 1. EGF levels measured in serum or plasma samples separated from the blood of 10 female and 10 male volunteers, collected into 6 different types of test tubes. The data represent the mean $\pm$ SD values of a total of 20 individuals (A) and the mean $\pm$ SD values of 10 female and 10 male individuals, separately (B). EGF level was undetectable in plasma samples obtained from K2EDTA-containing plastic tubes.

gel, and PP tubes ($p < 0.05$ for all comparisons). A cross-presentation of the statistical comparisons between various tube types is summarized in Table 1.

As an unexpected finding, we noticed that female volunteers showed statistically significant higher EGF levels than males; the EGF levels for both

genders differed significantly in relation to their sera collected and separated in glass ($p = 0.002$), PS ($p = 0.035$), vac-no gel ($p = 0.009$), and PP tubes ($p = 0.001$). The comparative data are summarized in Figure 1.

The difference in EGF levels between females and males was most prominent in PP tubes. The EGF level at $1/9$ diluted serum sample was non-detectable in 8 out of 10 male and in 1 out of 10 female volunteers ($p = 0.001$).

Difference in hEGF Levels of Female and Male Volunteers in Blood Collected in Polypropylene Tubes

Based on the statistically significant difference observed in hEGF levels measured in blood collected in PP tubes between female and male volunteers ($p = 0.001$), we designed another set of experiment, with a greater number of volunteers, to measure hEGF levels in sera separated in PP tubes. 17 female (31.2 ± 7.8 years of age) and 17 male (29.8 ± 5.4 years of age) volunteers participated in this experiment. After separation of serum from blood collected in PP tubes, EGF contents were measured at various dilutions. Under the same experimental conditions, female serum EGF contents (234.19 ± 172.9 pg/mL) were found to be significantly ($p = 0.014$) higher than those of male serum EGF contents (122.77 ± 105.5 pg/ml), as depicted in Figure 2.

Size Exclusion Chromatography of Platelet Lysate

It has been shown that the hEGF concentration in circulation is very low;[3] however, after $+4^{\circ}$C overnight storage in blood collecting tubes, we measured high levels of hEGF in sera but not in plasma of female and male volunteers. Therefore, we hypothesized that clotting activity continued during the storage of blood overnight and hEGF was released from the platelets by this process, since EGF release from platelets had been previously

Table 1. Statistical comparisons of EGF levels of sera obtained using different types of tubes

Tubes	Glass	PS	Vac-gel	Vac-no gel	PP
Glass		0.309	0.253	0.03^a	$<0.00001^a$
PS	0.309		0.041^a	0.0008^a	$<0.00001^a$
Vac-gel	0.253	0.041^a		0.221	$<0.00001^a$
Vac-no gel	0.03^a	0.0008^a	0.221		$<0.00001^a$
Plasma	$<0.00001^a$	$<0.00001^a$	$<0.00001^a$	$<0.00001^a$	$<0.00001^a$
PP	$<0.00001^a$	$<0.00001^a$	$<0.0001^a$	$<0.00001^a$	

[a]Asterix indicates a significant difference.

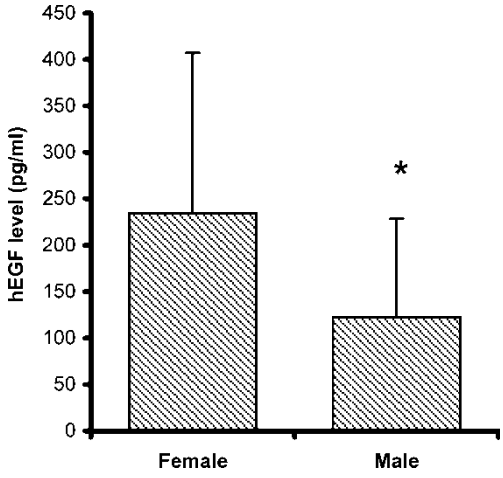

Figure 2. EGF levels measured in serum samples separated from the blood of 17 female and 17 male volunteers by collecting into polypropylene tubes. Asterix indicates a significant difference. The data represent the mean ± SD values of each gender group.

shown.[9–12] To confirm this, peripheral blood was collected from a male volunteer into a heparinized tube and platelets were isolated as described above. When the isolated platelets were lysed and loaded onto the TSKgel G3000SW column (Figure 3A), most of the immunoreactive fractions seemed to be eluted between 37–55 minutes of elution time (Figure 3B). In preliminary experiments, it was determined that serum proEGF and mature EGF eluted at 45–55 and 85–95 minutes of elution time, respectively. Therefore, it is reasonable to conclude that measurable EGF concentrations compared to human plasma might be related to EGF release from activated platelets in a processed form (i.e., 6 kDa) owing to clotting activity.

DISCUSSION

Epidermal growth factor (EGF) was first identified by Levi-Montalcini and Cohen[13] as a "tooth-lid factor," since it caused eye lid opening and incisor eruption when injected into neonatal mice. When its ability to promote epidermal growth and keratinisation was discovered, it was renamed as "EGF". EGF is a globular protein of 6.4 kDa consisting of 53 amino acids. Human EGF is synthesized as a prepro-protein of 1207 amino acids from which the factor (positioned 970-1023) is released by proteolytic cleavage.[14] Although the list of actions ascribed to EGF is too long, many aspects of its regulation, function, and physiological and pathological roles remain obscure.

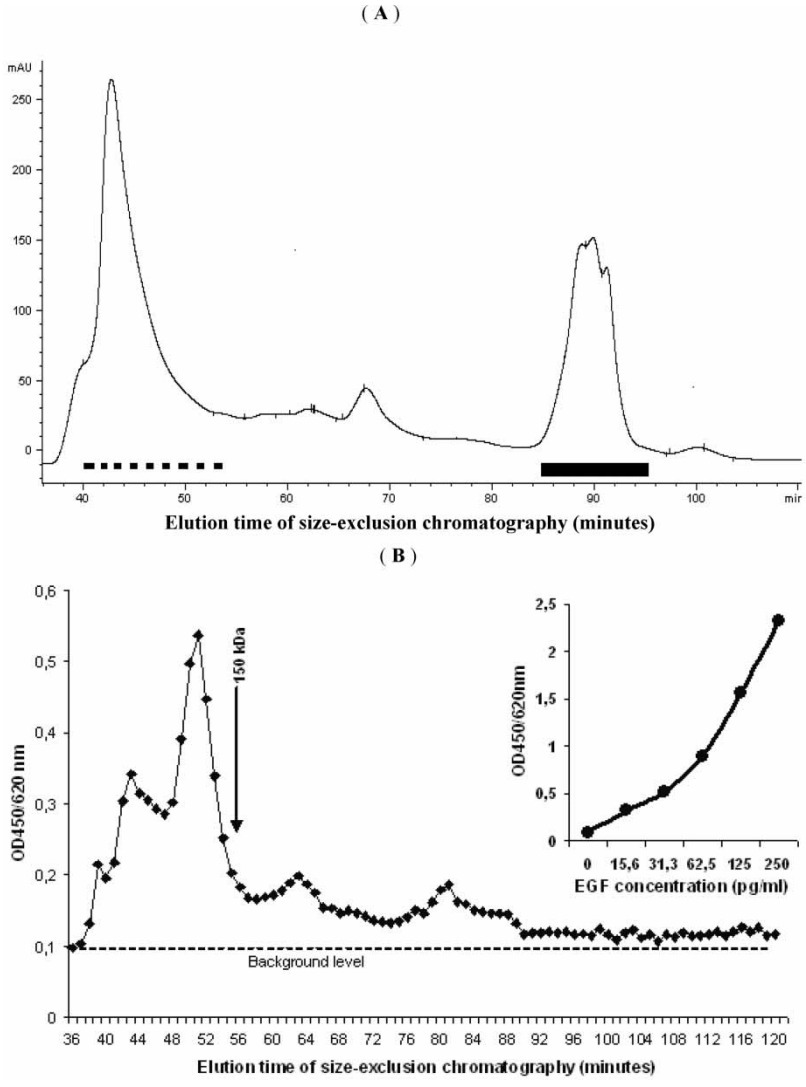

Figure 3. TSKgel G3000SW column fractionation of human platelet lysate. A 2 mL of freshly prepared platelet lysate was fractionated through column (A). Absorbance at 280 nm was recorded on line during elution of each fraction (A). Horizontal dotted line indicates the elution time of proEGF from human serum. According to the preliminary experiments, the fractions corresponding to the horizontal solid line represents the elution time of mature EGF from human serum. The eluted fractions were analyzed for EGF content (B). Data are the mean OD values of duplicate wells obtained using the hEGF ELISA. Horizontal dotted line in Fig. B demonstrates the background level of hEGF ELISA. The inlet demonstrates the standard curve for hEGF ELISA. Immunoreactive fractions were detected corresponding to molecular mass equal to and higher than 150 kDa.

Use of appropriately collected and stored test samples and sensitive and specific methods are very critical to immunoassays aimed to reliable measurement of cytokines and growth factors.[5,6] Biological variabilities, such as age, sex, and pre-analytical variabilities such as storage and processing can interfere with measurement of certain analytes in body fluids.

There are many studies in the literature about the effects of specimen handling, freeze-thawing, temperature, and other storage conditions, as well as the choice of anti-coagulants in antigen measurement.[4,7,15–21] Detected levels of cytokines might be affected by repeated freeze-thawing, by methods of storage and use of anticoagulant.[7,22,23]

However, effects of blood collecting tube types have been less frequently investigated, except for some toxicants, drugs, and hormones. Differing serum concentrations were measured for polychlorinated biphenyls, and for some hormones and tumour markers, when plastic and glass containers were compared.[24–26] Glass K3EDTA and plastic K2EDTA blood collecting tubes were compared for their storage-induced changes on platelet activation parameters; the difference was found to be of equal magnitude for both tube types.[27] Moreover, plain blood collecting tubes may have limitations, since complete separation of serum and blood cells may fail under certain conditions. In serum separator tubes, however, an inert gel moves to the serum-clot interface during centrifugation and provides a barrier between serum and clot, as well as reducing haemolysis.[28]

We noticed that the level of EGF was very low when measured immediately in plasma and serum samples which had been separated from cellular components directly after blood collection, but the level increased with time when the blood sample was kept for varying periods in test tubes for clotting (data not shown); therefore, we kept blood samples overnight at $+4^{\circ}C$ before centrifugation. We also noticed that the level of hEGF measured in serum or plasma varied with the type of blood collecting tube used when samples were kept in tubes overnight. So, we hypothesized that, for reliable measurements of hEGF, the type of tubes used for blood collection and blood clotting could have an important impact on the measured level of hEGF.

Based on these observations, and the deduced hypothesis, we designed and performed a few sets of experiments in which only the material of the blood-collecting tube differed. Blood was drawn from each volunteer at the same time to all tube types tested and this was valid for all participants at each set of experiments. By keeping all other possible pre-analytical conditions constant and equal for all participants, we attributed the differences in measured EGF levels directly to the material of the test tubes. Different EGF levels for the same participant could be explained by the effect of tube material on the clotting activity and, thus, release of EGF from platelets during activation.

Measured EGF levels in glass and polystyrene tubes did not differ significantly ($p > 0.05$), whereas, when EGF levels measured in glass and

polystyrene tubes were compared with the levels measured in the PP, a statistically significant difference was observed ($p < 0.05$). This finding points to a special concern, because PP tubes are widely used in laboratory practice, such as in Eppendorf tubes or in many blood collecting tubes. The finding that gel contained in serum-separator tubes did not affect the measured EGF content lets us suggest that acceleration of clot formation by such a kind of gel has no effect on the measurement of hEGF.

During these experiments, we also noted that the level of EGF was nondetectable in 8 of 10 male volunteers, while only in one out of ten female volunteers at 1/9 serum dilution in polypropylene tubes. There was a statistically significant difference ($p = 0.001$) between male and female volunteers in nondiluted serum as well. This difference in measured EGF levels for the two genders was also observed to be statistically significant for glass tubes ($p = 0.002$), polystyrene tubes ($p = 0.035$), and plastic tubes without clot activator gel ($p = 0.009$), with female hEGF levels being higher than those of males (Figure 1).

In order to confirm these unexpected data regarding the difference between genders, we designed another set of experiment with a larger number of volunteers (17 female and 17 male) to determine the serum EGF content collected and separated in polypropylene tubes (Figure 2). In previous studies, it had been shown that adult male plasma/serum EGF level was two-fold higher than female in mice, and testosterone treatment of female mice doubled serum EGF level[29] and that EGF level in tear fluid was higher in male compared to female patients;[30] contrary to these reports, we measured serum EGF levels significantly higher in female compared to male volunteers ($p = 0.014$). Our data regarding the higher serum EGF levels observed for females could be explained by the roles of some hormones reported previously as for progesterone up-regulating EGF expression[31] and, as for 17-beta estradiol enhancing heparin binding EGF-like growth factor production in human keratinocytes[32] and as well as with increasing EGF levels towards term.[33]

Using the ELISA system we developed for measurement of hEGF, hEGF levels were below detectable limits in all plasma samples of all volunteers who participated in this study. We thought this significant difference between serum and plasma levels might be due to hEGF in platelets, and that hEGF associated with platelets was released into serum after keeping blood overnight with the continuation of clotting activity due to possible influence of type of test tube. In order to indirectly confirm our hypothesis about the platelets being a source of hEGF in blood, we isolated platelets from heparinized blood and lyzed approximately 75×10^7 platelets. When the platelet lysate was loaded onto a size exclusion column (Fig. 3A), most of the immunoreactive fractions seemed to be located in fractions corresponding to preproEGF and/or proEGF (120–130 kDa) elution times (Fig. 3B). Therefore, we concluded that higher concentrations of EGF, compared to plasma sample, might be associated with platelets with the continuation of clotting activity.

Oka, et al.[12] had reported that hEGF was associated with blood platelets; they used immunoaffinity chromatography to extract hEGF from platelet-rich plasma. They indicated that hEGF was present in platelet-rich, but not in platelet-poor plasma and serum, and was found predominantly in the platelet fraction of whole blood. They showed that the concentration of immunoreactive hEGF in platelet-rich plasma exceeded that which was previously reported in plasma by Hirata, et al.[34] They thought this difference might be due to differences in procedures for collecting blood and preparing plasma.[12] Hwang, et al.[11] investigated the release of different fractions of epidermal growth factor from human platelets *in vitro* and noted preferential release of a 140 kDa fraction without substantial change in the content of the 67 kDa and 6 kDa fractions. When we loaded platelet lysate onto a defined column, we observed a prominent peak corresponding to 120–500 kDa, between 37–53 min (Figure 3A). We measured the hEGF content of eluates taken at 1 min intervals; we noted that the hEGF level in platelet-lysate started to increase at about the 40th min with the highest point of the peak at about the 49th min, and decreased to nearly baseline level at the 60th min (Figure 3B). The results suggested that the hEGF in circulation seemed to be also associated with platelets and that keeping blood overnight at $+4^{\circ}C$ caused continuation of blood coagulation which led to the release of hEGF from platelets.

CONCLUSION

As a conclusion, we want to emphasize that blood EGF levels are susceptible to changes in blood clotting, test tube type, and gender. All blood samples should be taken and stored in the same type of test tubes in order to make precise measurements, to avoid conflicting data regarding the human EGF measurement.

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