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Characterization of human epidermal growth factor in human serum and urine under native conditions

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Abstract

The objective of this study was to investigate the molecular nature of the human epidermal growth factor (EGF) in serum and urine samples of normal subjects. Recombinant EGF emerged as a single peak and did not interact with human IgG1 and albumin up to the concentration of 12 μg/ml. Freshly separated human serum contained only trace amounts of EGF. However, EGF appeared and increased in serum separated from blood after spontaneous overnight clotting. The authentic 6 kDa form of EGF made up nearly 40% of the total EGF in serum and revealed relatively homogeneous feature. The remaining immunoreactive fractions corresponded to 160 kDa proEGF. Immunoreactive EGF in blood seemed to be associated with the EGF release from platelets. TSKgel G3000SW chromatography of freshly-voided morning and day urines revealed that urine samples mainly contained two major form of EGF; a high-molecular-weight (HMW) and low-molecular-weight (LMW) forms. In the sense of molecular nature of EGF contents, morning urine was more heterogeneous than day urine of the same individuals. The LMW form of EGF in morning urine, in which its proportion was more than 90% of the total EGF, revealed further heterogeneous feature generally containing three to four different components.

Keywords: Epidermal growth factor; Serum; Urine; ELISA; Chromatography

1. Introduction

Epidermal growth factor (EGF) is a globular protein of nearly 6 kDa consisting of 53-amino acids [1]. Epidermal growth factor is originally discovered in the mouse submaxillary gland [2] and has been subsequently shown to stimulate the proliferation and differentiation of epidermal and epithelial cells and serves as a potent mitogen for a variety of cell types [3,4]. Human EGF is synthesized as a very long prepro-protein of 1168–1207 amino acids from which the factor itself (i.e. mature EGF, position 970–1023) is released by proteolytic cleavage [5–7]. This large molecule contains a hydrophobic domain allowing the precursor to be anchored in the plasma membrane [7]. However, the mechanism by which prepro-EGF is processed to mature EGF is not well understood. It was proposed that in cells incapable of cleaving this precursor, such as kidney cells, the membrane-bound form itself might also serve as a receptor for as yet unknown ligands [7,8]. Although epidermal growth factor is found in varying concentrations in milk, saliva, urine, and serum [9,10], there are conflicting reports in the literature regarding the exact nature of EGF molecules (i.e. proEGF or monomeric mature EGF) under native conditions [7,8,11–15].

Numerous evidences indicated that EGF and its receptor might play an important role in different clinical situations including carcinogenesis, chronic obstructive pulmonary disease, neonatal necrotizing enterocolitis, and skin inflammation and the high-molecular-weight form of EGF seemed to be related with the expression of the transformed phenotype [16–20]. Therefore, discriminative analysis of EGF, in the sense of molecular-weight form under native conditions, seemed to be important for investigating the relationship of EGF with certain types of pathological conditions.

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The relative amounts of high- and low-molecular-weight forms of EGF in urine have been debated, since different results have been obtained by different techniques. In order to address the problem it is important to quantitate the different molecular forms of EGF with a well-established method or combined methods.

The purpose of this study is to develop a processing-independent ELISA that is capable of quantitating different molecular forms of human EGF with equimolar potency and analyze proEGF and mature EGF in fractionated human serum and urine samples using a well-established size-exclusion chromatography technique.

2. Material and methods

2.1. Reagents

The following materials were purchased from commercial suppliers: RPMI-1640, L-glutamine, dimethyl sulfoxide (DMSO), streptavidine, recombinant human epidermal growth factor (rh-EGF) (Sigma, St. Louis, MO, USA); foetal calf serum (FCS, FCS was heat-inactivated by keeping at 56 °C for 30 min), bovine serum albumin (BSA, Fatty acid free) (PAA Laboratories GmbH, Linz, Austria); EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL 61105); streptavidine-horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA 94010); anti-human tumor necrosis factor alpha IgG1 human monoclonal antibody (Remicade™, Infliximab, 100 mg, Serial no: 03A032 Q, Schering-Plough Corporation, 2000 Galloping Hill Road Kenilworth, NJ); anti-human IL-6 IgG1κ (clone: 1A11a) mouse monoclonal antibody (mAb) and anti-hepatitis B surface antigen (HBsAg) "a" common epitope-specific IgG2a mouse mAb (clone: 4D1) were kindly provided by ImmunoGuide Laboratories (Erdem Biotechnology Ltd., Cyperpark, Bilkent, Ankara, Turkey).

2.2. Anti-hEGF monoclonal antibodies

Splenocytes of a female BALB/c mouse, immunized with rh-EGF, were fused with FO myeloma cells using polyethylene glycol. More than 50 hybridomas were produced and 10 out of them were selected and then further characterized. Hybridoma clones 3E12a (IgG1κ) and 2B6a (IgG1κ) were used as the source of capture and tracer antibodies, respectively. Determination of isotype was performed with a commercially available isotyping kit (ImmunoPure Monoclonal Antibody Isotyping Kit I (HRP/ABTS), Pierce Biotechnology, Inc. Rockford, USA).

2.3. Purification of monoclonal antibodies

Monoclonal antibodies specific for human EGF were purified from supernatants of hybridomas cultured in RPMI-1640 medium supplemented with 10% IgG-depleted FBS using Hi-Trap protein G column (1 ml MAbTrap G II Kit, Supelco Bellefonte, PA) attached to a high performance liquid chromatography (HPLC) system. Protein G-bound antibodies were eluted with 0.1 M citric acid pH 2.5 and then immediately neutralized with trizma. Eluted peak fraction was passed through Sephadex G-25 fine column (1 × 30 cm). Protein concentration was measured spectrophotometrically using bovine serum albumin (BSA) as the standard. Monoclonal antibodies were stored at −80 °C until use. Anti-hEGF 2B6a mAb, used as the tracer, was labeled with biotin (EZ-Link Sulfo-NHS-LC-Biotin, Pierce Biotechnology, Inc., Rockford, USA) according to the manufacturer's instructions.

2.4. Human serum and urine samples

Human blood samples were obtained from laboratory personnel with a written informed consent. Upon collection, the blood samples were alliquoted into two equal parts. One of them was allowed to clot overnight at 4 °C, the serum portion was then separated and analyzed after filtering through 0.2 μ. In order to obtain fresh serum, blood was immediately centrifuged, the plasma fraction transported to another clean tube and allowed to clot at 4 °C for 2 h and then analyzed after filtering through 0.2 μ. This serum was referred to as freshly separated serum. Urine specimens were collected into a sterile tube. The cellular debris was removed by centrifugation and then immediately analyzed after filtering through 0.2 μ. Day urine samples were collected nearly 8 h after the morning urine from the same individuals.

2.5. Size-exclusion chromatography

ProEGF and mature EGF contents of samples were analyzed using a HPLC (1100 Series, HPLC system, Agilent Technologies, GmbH, Waldbronn, Germany) consisting of a quarternary gradient pump (QuatPump G1311A), an eluent degas module (Degasser G1322A), a variable-wavelength detector (MWD G1365B), and a rheodyne sample injector (Man.inj G1328B) equipped with sample loops at varying volumes. The original chromatography software (ChemStation for LC 3D, Rev. A.08.03 Agilent Technologies (1990–2000) was used to program runs and perform data analysis. A TSKgel G3000SW-packed stainless steel column (21.5 mm × 300 mm, 13 μm particle size, TosoHaas, Japan) was attached to HPLC system. After equilibrating the column with mobile phase (phosphate buffered saline, PBS, 10 mM phosphate buffer and 154 mM NaCl, pH 7.4), 1 or 2 ml of fresh sample was loaded. The fractionation was performed at a constant flow rate of 1 ml/min at room temperature (RT). Column eluates were monitored by absorbance at 280 nm wavelength and 1 ml fractions were collected for ELISA. Each fraction was collected into polypropylene tubes at 1 min intervals. All column procedures were carried out at RT.