Journal of Chromatography, 568 (1991) 226–231
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5907

Short Communication

Variation of hydrophobicity of human urinary epidermal growth factor

KRISTINA PESONEN

Children's Hospital, University of Helsinki, Helsinki (Finland)

(First received April 18th, 1990; revised manuscript received March 4th, 1991)

ABSTRACT

Epidermal growth factor is present in human urine in large amounts, but its biological significance is not known. The results of this study indicate that the predominant 6000-dalton form of epidermal growth factor in human urine is divided by hydrophobic interaction chromatography into four fractions; only 3% of the total 6000-dalton epidermal growth factor coeluted with the biosynthetic epidermal growth factor and the rest was separated into three different peaks. These different forms may lack one or two amino or carboxy terminal amino acids from the 53 amino acids present in epidermal growth factor, or they may be products of deamidation or oxidation of amino acid(s). Further knowledge of these micromodifications of epidermal growth factor secreted in urine may reveal the origin and function of epidermal growth factor in humans.

INTRODUCTION

Epidermal growth factor (EGF) is a 53-amino acid (6000 dalton) polypeptide, which contains three disulphide bonds. It stimulates the growth of normal and malignant cells [1,2]. Several human tumours express the EGF receptor [3,4], which also binds another agonist, the transforming growth factor-α (TGF-α), which has 30% homology with EGF in amino acid sequence. In humans a high-molecular-mass EGF has been shown to be similar to or identical with the human brain tumour-associated urinary TGF-α [5]. EGF occurs in human body fluids in various concentrations [6]. In urine, the concentration of EGF is high, the 6000-dalton form predominating (*ca.* 95% of the total immunoreactive (ir) EGF) [6,7].

EGF may also vary in other respects than size. EGF-like material from human milk has been fractioned into four different forms by reversed-phase high-performance liquid chromatography (RP-HPLC), one of which is identical with β-urogastrone. They all compete with mouse EGF (mEGF) for the same placental receptor [8]. In human urine, eight different forms of EGF have been found by RP-HPLC: the intact 53-amino acid EGF, and forms lacking one, two or three carboxy terminal amino acids, as well as the respective oxidized forms [9]. RP-

0378-4347/91/$03.50 © 1991 Elsevier Science Publishers B.V.

HPLC analysis of recombinant EGF produced in yeast revealed that the predominant form, which was the most hydrophobic of the four fractions found, lacked the carboxy terminal arginine and was identical with γ-urogastrone. In addition, EGF lacking carboxy terminal arginine and leucine, as well as the respective oxidized forms were observed [10].

To study further the molecular diversity of human urinary EGF, a method was developed that separates the various forms of EGF from each other under non-denaturing conditions. The main molecular form of urinary EGF was first separated by gel permeation chromatography, and then analysed by hydrophobic interaction chromatography (HIC).

EXPERIMENTAL

Urine samples

Night urine of a healthy adult man was collected and concentrated in a dialyzer (M_r cut-off limit 2000) intended for treating patients with renal failure [11]. The concentrated urine was lyophilized.

Measurement of EGF

The concentration of EGF was measured by a time-resolved immunofluorimetric assay (TR-IFMA) [12]. In brief, a sandwich-type two-step solid-phase technique was used. The solid phase was prepared by coating wells in polystyrene microtiter strips with polyclonal anti-EGF antibody. As a tracer, monoclonal anti-EGF antibody labelled with Eu^{III} was used according to the instructions of the manufacturer (Wallac Oy, Turku, Finland), who donated the labelling reagents. The anti-EGF antibodies and the biosynthetic EGF used as standard were a kind gift from AmGen (Thousand Oaks, CA, USA).

Gel permeation chromatography

A Sephadex G 100 (Pharmacia, Uppsala, Sweden) column (85 cm × 1.6 cm I.D.) was packed using 0.1 M ammonium acetate (pH 6.0) as equilibration and elution buffer at 4°C. For M_r values the column was calibrated with Blue dextran 2000 (M_r $2 \cdot 10^6$ dalton, void volume), bovine serum albumin (BSA, 67 000 dalton), cytochrome C (12 400 dalton) and [^{125}I]EGF (6000 dalton). Lyophilized urine was reconstituted with 18 ml of elution buffer, applied to the column, and eluted by upward flow at 10 ml/h (LKB peristaltic pump, LKB, Bromma, Sweden); 6.4-ml fractions were collected (Redirac 2112, LKB). Their irEGF concentrations were measured, and fractions forming the main peak of EGF were pooled and lyophilized.

Size-exclusion high-performance liquid chromatography

The possible losses of irEGF during dialysis and concentration were controlled by measuring the total concentration of EGF and by chromatographing

the original sample and the concentrate on an Ultropac TSK G3000SW column (300 mm × 7.5 mm I.D.) equipped with a TSK SWP precolumn (75 mm × 7.5 mm I.D.). The apparatus consisted of an LKB 2150 HPLC pump, a Rheodyne 7125 injector (Cotati, CA, USA) and an LKB Redirac fraction collector. A 100-μl sample was injected and eluted at a flow-rate of 0.25 ml/min with 10 m*M* sodium phosphate buffer (pH 6.8), containing 0.1 *M* NaCl and 20% (v/v) acetonitrile. Fractions of 0.5 ml were collected and the acetonitrile was evaporated before determination of EGF. The column was calibrated with Blue dextran 2000, BSA, di-BSA (M_r 134 000 dalton) and [^{125}I]EGF.

Hydrophobic interaction chromatography (HIC)

The lyophilized sample from the Sephadex G 100 chromatography was reconstituted with 2 ml of 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.5 *M* Na_2SO_4, and chromatographed on a TSK Phenyl 5 PW column (75 mm × 8 mm I.D.; LKB). The apparatus consisted of a Waters Assoc. two-pump HPLC system (Milford, MA, USA), a Rheodyne 7125 injector with 1-ml sample loop and a fraction collector (LKB Redirac). Sample of 2 ml were injected (2 × 1 ml) and eluted with 20 m*M* sodium phosphate buffer (pH 7.0), with a linear 30-min gradient from 0.5 to 0 *M* Na_2SO_4 at 0.8 ml/min. Fractions of 1.2 ml were collected and assayed for EGF.

RESULTS

Urine (2.65 l) was concentrated to 400 ml (6.6-fold). The concentration of EGF was 29.1 ng/ml in the original urine and 110 ng/ml in the concentrate. Thus the recovery of EGF was 57%. In the original and the concentrated samples, the 6000-dalton EGF formed 97 and 95%, respectively, of the total immunoreactive EGF as revealed by size-exclusion HPLC. Recovery of EGF in the chromatographic step was 110%. After preparative gel chromatography on Sephadex G 100, the 6000-dalton EGF formed 95% of the immunoreactivity.

In TR-IFMA the recovery of authentic EGF added to urine was 98%. Inter- and intra-assay variations were 7 and 6%, respectively, and the concentration range was 3–5000 pg/ml.

In HIC irEGF variants of *ca.* 6000 daltons eluted at four different salt concentrations (Fig. 1). The relative amounts of EGF variants corresponding to the peaks I to IV were 22, 5.3, 3.0 and 70%, respectively, of the total 6000-dalton EGF. The biosynthetic EGF coeluted with peak III.

DISCUSSION

HIC is a relatively new analytical technique, analogous to reversed-phase chromatography (RPC). Both methods utilize hydrophobic ligands as the bonded phase of the support, which binds hydrophobic structures in aqueous solution.

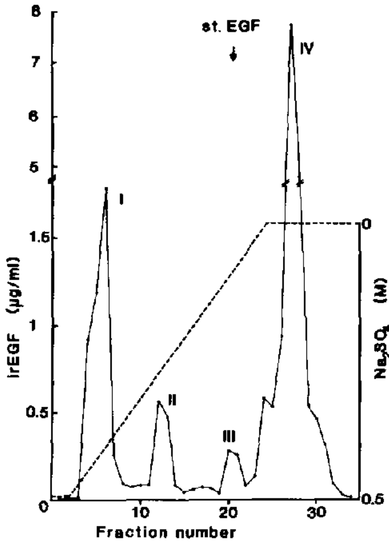

Fig. 1. Concentrated 6000-dalton fraction from gel permeation chromatography on Sephadex G 100 analysed by HIC. Two 1-ml samples were injected and eluted with a Na_2SO_4 gradient from 0.5 to 0 *M* in 30 min in 20 m*M* sodium phosphate buffer (pH 7.0). A flow-rate of 0.8 ml/min was used, and fractions of 1.2 ml were collected. The concentration of EGF in the fractions is plotted against the fraction number. The salt gradient is shown as a dashed line.

The binding force between a protein and the hydrophobic support depends on the hydrophobic areas on the surface of the protein, and these are specific for each protein. In HIC the binding force can be modified by certain salts, such as sulphates and phosphates. This method has the advantage that compounds such as proteins preserve their biological activity. In contrast, the organic solvents used as modifiers in RPC denature proteins. In HIC a decreasing salt gradient is used, and the compounds to be separated are eluted from the column in order of increasing hydrophobicity.

During the first concentration step, *ca.* 40% of the original irEGF was lost. However, since the proportion of the 6000-dalton irEGF was similar before and after the concentration step, it is evident that different types of irEGF were recovered to similar extents.

These results revealed an unexpected hydrophobic heterogeneity of human urinary EGF. The predominant 6000-dalton fraction was eluted in four peaks and, interestingly, the biosynthetic EGF coeluted with the quantitatively smallest of them. The structures of these EGFs are not known, but Mount *et al.* [9] have reported the existence of EGF molecules lacking one, two or three carboxy terminal amino acids in human urine. Moreover, mouse 53-amino acid EGF, which resembles human EGF in many respects, exists as des-Asn^1-EGF [13,14], as a deamidated (isoaspartyl^1) form and as des-Asn^1-Ser^2-EGF [15]. Even some com-

mercial mEGF standards, though reported to be homogeneous, contain components lacking one or two amino terminal amino acids [16]. On the basis of these findings it can be assumed that the hydrophobic heterogeneity of human urinary 6000-dalton EGF is due to deletions or modifications of one or a few amino acids.

The concentration of EGF in human urine is high, but no physiological significance of urinary EGF is known. This study demonstrates that only 3% of urinary EGF is identical with authentic EGF. The effect of minor modifications on the biological activity of EGF is not known. As regards another small biologically active peptide, endothelin, even very small changes in structures, such as the change or deletion of a single amino acid, may reduce the potency by two or three orders of magnitude [17]. If this also holds for EGF, it is possible that a large proportion of the urinary EGF is not biologically active. The application of HIC to the separation of the different forms of urinary EGF, described here for the first time, will allow further studies of their biological activity, which would not be possible after denaturing RPC.

CONCLUSION

Human urinary EGF shows a remarkable heterogeneity in its hydrophobicity. This is suggested to be due to deletions or modifications of one or a few amino acids.

ACKNOWLEDGEMENTS

This study was supported by grants from the Academy of Finland and the Sigrid Juselius Foundation. We thank Ms. Marjatta Vallas for excellent technical assistance and Ms. Jean Margaret Perttunen for correcting the language.

REFERENCES

1 G. Carpenter and S. Cohen, *Ann. Rev. Biochem.*, 48 (1979) 193.
2 M. D. Hollenberg, *Vitam. Horm.*, 37 (1979) 69.
3 B. Gusterson, G. Cowley, J. McIlhinney, B. Ozanne, C. Fisher and B. Reeves, *Int. J. Cancer*, 36 (1985) 689.
4 T. A. Liberman, H. R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, M.D. Waterfield, A. Ullrich and J. Schlessinger, *Nature (London)*, 313 (1985) 144.
5 K. Strömberg, R. Hudgins, L. Dorman, L. Henderson, R. Sowder, B. Sherrell, C. Mount and D. Orth, *Cancer Res.*, 47 (1987) 1190.
6 K. Pesonen, L. Viinikka, A. Koskimies, A. Banks, M. Nicolson and J. Perheentupa, *Life Sci.*, 40 (1987) 2489.
7 Y. Hirata and D. Orth, *J. Clin. Endocrin. Metab.*, 48 (1979) 673.
8 P. Petrides, M. Hosang, E. Shooter, F. Esch and P. Böhlen, *FEBS Lett.*, 187 (1985) 89.
9 C. Mount, T. Lukas and D. Orth, *Arch. Biochem. Biophys.*, 240 (1985) 33.
10 C. George-Nascimento, A. Gyenes, M. Halloran, J. Merryweather, P. Valenzuela, K. Steimer, F. Masiarz and A. Randolph, *Biochemistry*, 27 (1988) 797.

11 U.-H. Stenman, K. Pesonen and M.-L. Huhtala, *Anal. Biochem.*, 123 (1982) 291.
12 K. Pesonen, H. Alfthan, U.-H. Stenman, L. Viinikka and J. Perheentupa, *Anal. Biochem.*, 157 (1986) 208.
13 P. Petrides, P. Böhlen and J. Shively, *Biochem. Biophys. Res. Commun.*, 125 (1984) 218.
14 R. DiAugustine, M. Walker, D. Klapper, R. Grove, W. Willis, D. Harvan and O. Hernandez, *J. Biol. Chem.*, 260 (1985) 2807.
15 R. DiAugustine, B. Gibson, W. Aberth, M. Kelly, C. Ferrua, Y. Tomooka, C. Brown and M. Walker, *Anal. Biochem.*, 165 (1987) 420.
16 M. Riemen, R. Wegrzyn, A. Baker, W. Hurni, C. Bennett, A. Oliff and R. Stein, *Peptides*, 8 (1987) 877.
17 S. Kimura, Y. Kasuya, T. Sawamura, O. Shinmi, Y. Sugita, M. Yanagisawa, K. Goto and T. Masaki, *Biochem. Biophys. Res. Commun.*, 156 (1988) 1182.