Occurrence of High Molecular Weight EGF Complexes in Human Milk

Norihiro Azuma, Eri Hesaka, Shuichi Kaminogawa and Kunio Yamauchi

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received November 11, 1988

A survey was conducted of the cell-growth-promoting factors in human milk using anti-human EGF antiserum and by a DNA synthesis assay in BALB/c mouse 3T3 embryo fibroblast cells. Three fractions having affinity toward anti-human EGF antibody were found by gel filtration on Sephacryl S-300. The fraction eluted at the void volume and the one with a molecular weight of about 70,000 both showed weak DNA synthesis stimulatory activity. On the other hand, the one with a molecular weight of about 6,000 highly stimulated DNA synthesis. This fraction was thought to contain EGF, whose molecular weight is about 6,000. Rechromatography by gel permeation again separated both the two high molecular weight peaks into 3 peaks and produced similar chromatograms. The results suggest that these 3 fractions may have been in an apparent equilibrium state. Considering that the EGF-fraction of molecular weight 6,000 did not show self-association, it is presumed that an EGF binding protein exists in human milk and forms higher molecular weight EGF complexes.

The presence of cell-growth-promoting or colony-stimulating factors in human milk has been demonstrated,1~5 and some of these factors have been isolated and characterized. Among them, the epidermal growth factor (EGF) has been identified as a major growth-promoting agent using anti-human EGF/β-urogastrone antibody.2,3 Recently, EGF in human milk has been isolated, and its N-terminal amino acid sequence was determined.9 The partial sequence of EGF from human milk was identical with that of the counterpart of EGF/β-urogastrone originating from human urine.10

Although the biological role of EGF is unclear, biological functions other than being a potent mitogen for various cells and an inhibitor of gastric acid secretion have been demonstrated by many investigators. It has been reported that EGF develops the mammary gland and milk production,11 prevents cysteamine-induced duodenal ulcer formation,12 and accelerates the process of wound repair.13,14

It has also been demonstrated that a part of EGF in mouse submaxillary gland exists as an EGF-arginine esterase complex (about 74 k daltons (kD)).17~19 This complex has been demonstrated to dissociate at a low or high pH level, and in a high salt concentration. High molecular weight EGF (about 28 ~ 33 kD) has also been found in human urine,15 which, unlike mouse EGF-arginine esterase complex, did not dissociate below pH 5, but could be converted by the action of arginine esterase16 to a low molecular weight EGF with 6 kD. The EGF in human milk, on the other hand, has been found to have size and charge heterogeneity,17 although the presence of EGF in a high molecular weight form was not demonstrated.

In this study, we report the presence of a high molecular weight form of EGF in human milk. This was recovered by gel filtration as three fractions, which are recognized by anti-human EGF (of urinary origin) antibody, but had only slight DNA synthesis-stimulatory activity in BALB/c 3T3 fibroblast cells. The
formation of high molecular weight EGF in human milk is not similar to either mouse EGF-arginine esterase complex\textsuperscript{18}) or to high molecular weight EGF of urinary origin.\textsuperscript{15,16)}

**Materials and Methods**

*Human skim milk.* Human milk samples were provided by a healthy mother 3 months after parturition. Each sample was immediately defatted by centrifugation at $1000 \times g$ and $10^\circ C$ for 15 min, and the skim milk was directly filtered on a gel without freezing or lyophilizing.

*Gel filtration on Sephacryl S-300.* Ten ml of human skim milk was put on a column (2 $\times$ 80 cm) of Sephacryl S-300 (Pharmacia Fine Chemicals) that had been equilibrated with 10 mM phosphate buffer containing 100 mM NaCl at pH 7.0. Elution was done with the same buffer at 4$^\circ C$ and at a flow rate of 15 ml/hr. The eluent was monitored at 280 nm and 3.5 ml-portions were collected. Each portion was submitted to a DNA synthesis assay and immunoassay with anti-hEGF antiserum (see details later), and active fractions were collected and lyophilized after dialyzing against water.

*Gel permeation chromatography (GPC) on TSK gel G3000 SW.* Three mg of each fraction separated by gel filtration on Sephacryl S-300 was dissolved in 500 $\mu l$ of the same phosphate buffer already used above and put on a TSK gel G3000 SW column (Toyo Soda) coupled to a Shimadzu LC 4A liquid chromatograph. Elution was done with the same buffer at room temperature and a flow rate of 0.5 ml/min. The eluent was monitored at 230 nm and 1 ml-portions were collected, each portion being submitted to a DNA synthesis assay and immunoassay (see the details later).

*Reverse phase high performance liquid chromatography (RP-HPLC).* The fractions obtained by GPC were concentrated to 2 ml by ultrafiltration and chromatographed by RP-HPLC, using a Shimadzu 4A liquid chromatograph with a high-pore C4 column (SSC-SC4, Senshu Kagaku). Chromatography was done at 40$^\circ C$ by linear-gradient elution of acetonitrile in 0.1% trifluoroacetate at a flow rate of 1 ml/min [from 15% to 75% acetonitrile in 40 min].

*Enzyme-linked immunosorbent assay (ELISA).* ELISA was done using alkaline phosphatase as the antigen-enzyme conjugate, by the method of Miedema et al.\textsuperscript{20)} Rabbit anti-human EGF (of urinary origin) antiserum and human EGF biosynthesized by Escherichia coli were purchased from Wakunaga Pharmaceutical Co., Ltd.

*DNA synthesis assay.* DNA synthesis in mouse BALB/c 3T3-3K embryo fibroblast cells (a gift from Professor K. Nishikawa of Kanazawa Medical University) was evaluated from the incorporation of $^3$H-thymidine by the method of Nishikawa.\textsuperscript{21)} In brief, 3T3 cells at the subconfluent stage were suspended in Dulbecco’s modified Eagle’s minimum medium (DMEM, Nissui Pharmaceutical Co., Ltd.) which contained 3% fetal bovine serum (FBS, Gibco). The suspension containing 2 $\times$ 10$^6$ cells/ml was seeded into a 48-well plate (Corning). After this was incubated for 5 hr, the medium was replaced with 0.2% FBS-containing DMEM and incubated for 48 hr more. To the culture were then added the test materials, and 16 hr later, $^3$H-thymidine. After a 3 hr incubation, the $^3$H-thymidine incorporated into DNA was counted. Incubations were all at 37$^\circ C$ in a 5% CO$_2$-containing atmosphere. Test materials were added to the medium after sterilizing by filtration with a 0.45 $\mu m$ pore sized cellulose acetate filter (Toyo Roshi Kaisha Ltd.).

**Results**

**Chromatographic distribution of EGF in human milk**

When human skim was filtered on a gel, Sephacryl S-300, DNA synthesis stimulatory activity and affinity toward anti-hEGF antibody were found in 3 fractions (Fig. 1), both these activities being in each eluted fraction. These fractions were eluted at the void volume, next to lactoferrin and after $\alpha$-lactalbumin, and are termed Frs. I, II, and III, respectively. The molecular weights of the latter two were estimated to be about 70,000 and 6,000, respectively, from the elution volumes of lactoferrin, $\alpha$-lactalbumin, and EGF from human

| Table I. COMPARISON OF ACTIVITIES AMONG FRACTIONS HAVING CROSS-REACTIVITY TOWARDS ANTI-hEGF ANTISERUM OBTAINED BY GEL FILTRATION ON SEPHACRYL S-300 |
|-----------------|-----------------|-----------------|
| Fraction | Relative amount$^a$ | Relative activity$^b$ |
| Fr. I | 60.5 | 5.5 |
| Fr. II | 43.7 | 20.4 |
| Fr. III | 100 | 100 |

$^a$ Relative amount is expressed as the peak area of the ELISA titer profile in Fig. 1. The value for Fr. III is taken as 100.

$^b$ Relative activity is expressed as the ratio of DNA synthesis stimulatory activity (the peak area of the $^3$H-thymidine incorporation profile in Fig. 1) to the amount of EGF. The value for Fr. III is taken as 100.
High Molecular Weight EGF in Human Milk

Fig. 1. Gel Filtration Profile of Human Skim Milk on Sephacryl S-300.
A DNA-synthesis assay (--\(\triangle\)--) and ELISA with anti-human EGF antiserum (\(--\bullet--\)) were done on every other portion. \(\cdots\) shows the protein concentration monitored at 280 nm.
The arrows indicate the peaks of lactoferrin (\(\downarrow\)), \(\alpha\)-lactalbumin (\(\downarrow\)), and EGF from human urine (\(\uparrow\)).

Fig. 2. Gel Permeation Chromatogram of Fr. I Obtained by Gel Filtration on Sephacryl S-300 on TSK gel G3000 SW (A) and the Rechromatogram of Fr. I-a (B).
Each portion was submitted to ELISA (\(--\bullet--\)) and/or a DNA synthesis assay (--\(\triangle\)--). \(\cdots\) shows the protein concentration monitored at 230 nm.

In Table I are shown the relative DNA-synthesis stimulatory activities of these fractions. Fraction II seemed to contain a mammary-derived growth factor, the molecular weight of which was estimated to be 62,000. The second \(^3\)H-thymidine-incorporation peak
in Fr. II-b in Fig. 3A seemed to correspond to that of the mammary-derived growth factor, because its molecular weight was about 60,000 and it was not recognized by anti-human EGF antibody. Therefore, the DNA synthesis-stimulating activity of Fr. II is thought to have been due to the combined effects of the material, which was recognized by anti-hEGF antibody, and the mammary-derived growth factor.

As the elution volume of Fr. III was equal to that of EGF, and as EGF has been found to be a major growth-promoting agent in milk, Fr. III is thought to have been the fraction containing EGF. Therefore, the relative activity in the table is expressed by taking the value of Fr. III as 100. The concentration of EGF in Fr. III estimated by ELISA was calculated to be about 12 μg/l of skim milk.

**EGF in high molecular weight form**

To clarify whether the high molecular weight fractions that had immunoreactivity with anti-human EGF antiserum were really related to EGF, and to test the possibility that these materials might be a precursors of EGF, Frs. I and II were further submitted to the following experiments.

Figure 2A shows a typical GPC profile of Fr. I obtained by gel filtration on Sephacryl S-300. Fraction I was separated into three fractions (I-a, I-b, and I-c) that stimulated DNA synthesis and immunoreactibility with anti-hEGF. When each fraction was filtered on Sephacryl S-300 gel, Frs. I-a, I-b, and I-c were eluted at the identical positions to those of Frs. I, II, and III, respectively. As shown in Fig. 2B, rechromatography of Fr. I-a yielded the two fractions whose elution volumes were
Fig. 4. Gel Permeation Chromatogram of Fr. III Obtained by Gel Filtration on Sephacryl S-300 on TSK gel G3000 SW.
Each portion was submitted to ELISA (---●---) and DNA synthesis assay (---△---). ······ shows the protein concentration monitored at 230 nm.

Fig. 5. RP-HPLC Patterns of High Molecular Weight EGF Fractions Obtained by GPC on TSK gel G3000 SW in Figs. 2 and 3 on SSC-SC4 Column.
Each portion was submitted to ELISA with anti-hEGF antiserum (---●---). ······ shows the protein concentration monitored at 230 nm.
A, Fr. I-a; B, Fr. I-b; C, Fr. II-a; D, Fr. II-b. The arrow indicates the retention time of 6kD EGF.
identical to those of Frs. I-b and I-c.

In Fig. 3A is shown the GPC profile of Fr. II. From Fr. II were released both higher and lower molecular weight materials (Frs. II-a and II-c) corresponding to Frs. I and III in Fig. 1, respectively. Rechromatography of Fr. II-b (Fig. 3B) again produced the higher and lower molecular weight materials corresponding to Frs. II-a and II-c in Fig. 3A.

On the other hand, Fr. III did not change its chromatographic behavior upon repetition of GPC (Fig. 4). The retention time of the material recognized by anti-hEGF antibody corresponded to Fr. I-c, Fr. II-c, and to standard human EGF itself.

In Fig. 5 are shown RP-HPLC patterns of the high molecular weight EGF fractions from Frs. I and II. All the fractions contained material which was recognized by anti-hEGF antibody in a greater or lesser degree and whose retention time was identical with that of standard human EGF.

Survey of the hEGF-binding protein

Assuming that there existed an EGF-binding protein which was specific for EGF, and that such a protein had a region homologous to the EGF-receptor at least around the binding site, we used the high molecular weight EGF fraction (Fr. II obtained by gel filtration on Sephacryl S-300) in an enzyme-linked immunosorbent assay, using a monoclonal antibody to human EGF receptor that inhibits EGF binding to its receptor, produced by Oncogene Science Inc. The result revealed that this fraction contained a certain substance that was recognized by the anti-hEGF receptor antibody. For further confirmation, the high molecular weight EGF fraction was collected and put into a reverse-phase HPLC equipped with an Aquapore Butyl column (300 Å pore size, 4.6 mm x 6 cm, Brownlee Labs). About 1 mg of the fraction was dissolved in 1% SDS solution in 0.1% TFA and used in HPLC. Elution was done with a linear gradient of acetonitrile in 0.1% TFA from 0 to 30% in 2 min, and from 30 to 50% in another 20 min at a flow rate of 2 ml/min. Each fraction was collected manually and concentrated, and then used for ELISA using anti-EGF receptor antibodies. As shown in Fig. 6, the material recognized by anti-EGF receptor antibody was eluted with about 36% acetonitrile (a retention time of around 13 min) and this material was also recognized by anti-EGF serum. This substance has not purified and identified, but it may be an EGF receptor or its degraded product, and is possibly related to the formation of the high molecular weight EGF.

Discussion

In human milk, three forms of EGF with different molecular weights were detected by an enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human EGF antiserum; these were Fr. I eluted at the void volume, Fr. II with a molecular weight of about 70,000, and Fr. III eluted at the same volume as that of standard EGF. In Fr. III was found the greatest DNA synthesis-stimulatory activity in human milk. Considering that the major growth promoting
High Molecular Weight EGF in Human Milk

agent in milk is EGF, as reported by Carpenter,\(^2\) the immunoreactive agent in Fr. III is thought to have been EGF itself.

Fractions I and II only slightly stimulated DNA synthesis in BALB/c 3T3 cells, although the content of EGF in Fr. I and Fr. II as estimated by ELISA amounted to about 60% and 44% of that in Fr. III, respectively (Table I). Rechromatography by gel filtration seemed to indicate that the high molecular weight complex dissociated, releasing a material of molecular weight 6,000 that is considered to have been EGF itself.

The size heterogeneity of human EGF in urine has been discussed,\(^15,16\) in comparison with that of mouse EGF.\(^17\sim19\) It was demonstrated that high molecular weight EGF in human urine (about 28 ~ 33 kD) was converted into low molecular weight EGF by treating with mouse EGF-associating arginine esterase,\(^16\) with which mouse EGF formed a high molecular weight complex (about 74 kD), consisting of two molecules of EGF (6 kD) and two molecules of arginine esterase (29 kD).\(^17\sim19\) This complex could be readily and reversibly dissociated by gel filtration at below pH 5 or above pH 8, and by anion exchange chromatography when the NaCl concentration was increased at pH 7.5.\(^17\) However, the high molecular weight hEGF in urine did not dissociate under such conditions and was thought to possibly be a precursor of EGF.\(^16\)

On the other hand, our high molecular weight hEGF in milk dissociated both at neutral pH and at low pH levels, as well as in a high or low salt concentration (data not shown). Furthermore, the low molecular weight EGF in Fr. III dissociated via an intermediate complex of molecular weight 70,000 (Fr. II) from the large complex in Fr. I. The results of rechromatography of Frs. I and II suggest that they were in an apparent equilibrium state, although the low molecular weight material in Fr. III or those dissociated from Frs. I and II would never form a high molecular weight complex. These results infer that EGF in high molecular weight forms in human milk is not a precursor like that found in human urine, but is a complex with a binding protein, which is thought to be different from the EGF-arginine esterase complex in mouse submandibular. It is likely that EGF of molecular weight 6,000 in human milk forms a complex of molecular weight 70,000 with a binding protein, and that this complex further associates to form a very large EGF complex, which is eluted at the void volume on Sephacryl S-300 or on TSKgel G3000 SW chromatography. The substance separated by reverse-phase HPLC and recognized by anti-human EGF receptor antibody in Fig. 6 may be important in the formation of high molecular weight EGF complex.

DNA synthesis-stimulatory activity in Frs. I and II is thought to be attributed to EGF which was dissociated from these high molecular weight complexes. From a different point of view, it can be said that the DNA synthesis-stimulatory activity of EGF was reduced by forming a high molecular weight complex to about 5.5% in Fr. I and about 20% in Fr. II when compared with that in Fr. III (Table I). The apparent equilibrium state of the different formation of EGF may regulate the concentration of EGF activity in human milk.

It seems necessary to postulate the presence of a binding protein to explain the formation of high molecular weight EGF. However, the entity of this binding protein has not yet been grasped, although its isolation and characterization is now under investigation.

**Acknowledgments.** We are grateful to Professor Katsuzo Nishikawa of Kanazawa Medical University for the gift of BALB/c 3T3 cells and also helpful discussions and encouragement. We thank Dr. Masatsugu Ueda and Dr. Kunihisa Akai of Snow brand Milk Products Co., Ltd., and Dr. Shintaro Nomura of the Institute of Applied Microbiology at the University of Tokyo for technical advice and useful discussions.

**References**


3) Y. Hirata, T. Nishinuma, M. Uchihashi and T.