Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 230-235

EXPRESSION OF ERYTHROID DIFFERENTIATION FACTOR (EDF) IN CHINESE HAMSTER OVARY CELLS

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Received January 8, 1988

Summary: Plasmid DNA containing EDF subunit cDNA and mouse dihydrofolate reductase (DHFR) cDNA was transfected into CHO DHFR$^-$ cells by the calcium-phosphate method. DHFR positive transformants secreted recombinant EDF (r-EDF) constitutively in an active form and accumulated it in the conditioned medium. Furthermore, cells which were resistant to methotrexate (MTX : 0.5μM) secreted r-EDF up to 1μg/ml. r-EDF was identical to natural EDF (n-EDF) produced by human acute monocytic leukemia cell line, THP-1, as regards its dimeric structure and a biological activity.

Erythroid Differentiation Factor (EDF)(1) is secreted into the conditioned medium of TPA (tetradecanoylphorbol-13-acetate)-treated THP-1 cells (human acute monocytic leukemia cell line) (2). It shows differentiation-inducing activity and causes the differentiation of mouse Friend leukemia cells into more mature cells. EDF is a homodimer protein (monomeric MW, 15.5Kd) crosslinked by disulfide bonds. This structure is necessary for its activity because its activity is lost in reducing conditions.

EDF subunit cDNA had been isolated and sequenced(3). From the nucleotide sequence of EDF subunit cDNA, EDF subunit consisted of 116 amino acid residues, which was derived through processing from a large precursor (MW 42Kd), and the processing site of EDF precursor in THP-1 cells was after the five consecutive arginines, by which EDF subunit was preceded.

0006-291X/88 $1.50

EDF subunit cDNA was completely identical with human Inhibin-β_A cDNA (4), which was isolated from human ovary cDNA libraries. Inhibin is a specific and potent polypeptide inhibitor of follicle-stimulating hormone (FSH) and consists of α- and β_A-subunits. Recently a homodimer of Inhibin-β_A subunits, named FSH releasing protein (FRP)(5), was discovered from porcine follicular fluid. Both EDF and FRP were known to stimulate the release of FSH from cultured anterior pituitary cells.

To study biological functions in living body, overproduction of EDF was necessary. From the viewpoint of EDF structure, we attempted to express EDF as an active form using a CHO DHFR$^-$ cell/DHFR cDNA containing plasmid system.

Materials and Methods

Construction of the EDF expression vector (pSD(x)/EDF)

EDF subunit cDNA was inserted into the XhoI site of pSD(X)(6) in the ordinary direction, i.e., between the SV40 early promoter and the poly(A) signal (designated as pSD(X)/EDF).

Transfection of pSD(X)/EDF into CHO DHFR$^-$ cells

Ten micrograms of the constructed plasmid, pSD(X)/EDF, was transfected into CHO DHFR$^-$ cells by the calcium phosphate method (7), and transformants were selected after 2 weeks at 37 °C in a selection medium, which did not contain ribonucleoside and deoxyribonucleoside (MEM ALPHA medium CAT no. 410-2000, GIBCO).

Isolation of MTX-resistant transformants

First transformants were grown in 0.1μM MTX (methotrexate) containing selection medium, and the medium was changed every 3 days until MTX-resistant transformants appeared in this medium. According to the same procedure, 0.5μM resistant transformants were isolated from the 0.1μM MTX resistant transformants. The 0.5μM resistant transformants were then mono-cell isolated.

Northern blot analysis

Transformants were grown in 75 cm^2 flasks (about 1 X10^7 cells) and the total RNA was isolated from the cells by guanidium thiocyanate/ cesium chloride centrifugation(8). Ten micrograms of the total RNA was fractionated on a 1.2% formaldehyde gel(9), transferred onto a nitrocellulose filter and then hybridized with a [^{32}P]dCTP labelled probe.

Western blot analysis

Conditioned medium, which contained 10,000 EDF units, was concentrated using a Centrisart 1 (cut-off, 10,000; Sartorius). SDS-Polyacrylamide gel electrophoresis was carried out under reducing conditions and then Western blot analysis was performed. Blots were exposed to rabbit polyclonal antibody to EDF followed by goat anti-rabbit horse radish peroxidase conjugate and substrate.

Determination of the N-terminal amino acid sequence

EDF was purified from the 0.5μM MTX-resistant cell conditioned medium as described in the previous paper(1). Microsequence analyses were performed with an applied Biosystems 470A gas phase protein sequencer in conjunction with a 120A phenylthiohydantoin (PTH)-amino acid analyzer.

Assay of EDF activity

EDF producing cells were grown well in a 25cm^2 flask. After removing the conditioned medium, 10ml of the selection medium was added and cultured for 3 days. The conditioned medium was assayed for EDF activity according to the previous paper(1). The unit of EDF was defined as a mimimum effective dose to Friend cells.

Results and Discussion

The pSD(X)/EDF plasmid, which contained DHFR cDNA and EDF subunit cDNA, was constructed to express EDF constitutively (Fig.1). This pSD(X)/EDF plasmid was transfected into CHO DHFR$^-$ cells using calcium phosphate method. Transformants (DHFR$^+$) which were transfected with pSD(X)/EDF secreted EDF into the conditioned medium (60 U/ml) constitutively, whereas CHO DHFR$^-$ cells and pSD(X)-transfected cells secreted none (Table 1).

Transformants were grown in selection medium containing 0.5μM MTX, and the resulting 0.5μM-MTX resistant transformants

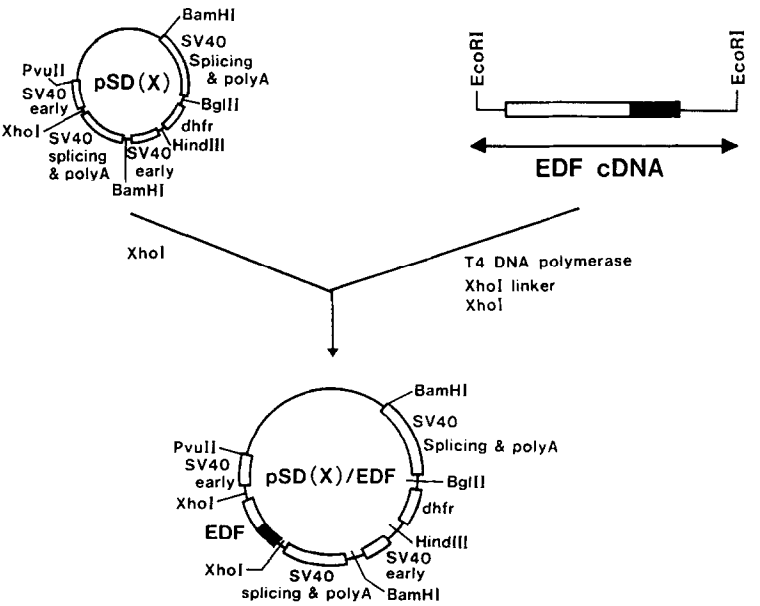


Fig. 1. Construction of EDF expression vector pSD(X)/EDF in CHO cells. EDF cDNA and DHFR cDNA were regulated under the control of the SV40 early promoter.

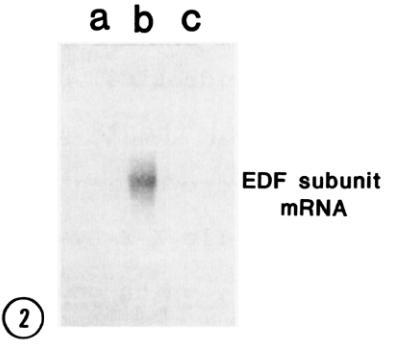
Table 1. Production of EDF.

Cell	Plasmid	MTX conc.(μM)	EDF activity (U/ml)
CHO DHFR⁻	--	---	---
CHO DHFR⁻	pSD(X)	---	---
CHO DHFR⁻	pSD(X)/EDF	---	60
CHO DHFR⁻	pSD(X)/EDF	0.5	2000
THP-1	--	---	250

The conditioned medium, for 3 days, after confluent culturing was assayed. In the case of THP-1, the conditioned medium, for 5 days, after TPA (100ng/ml) induction was assayed.

were found to secrete 2000 U/ml(coresponding to 1μg/ml) EDF into the conditioned medium. This productivity was 30 times higher than that of the early transformants. In the 0.5μM-MTX resistant transformants, overproduction of EDF subunit mRNA was detected, whereas in the early transformants, only minimal levels of EDF subunit mRNA were detected. No EDF subunit mRNA was detected in CHO DHFR⁻ cells (Fig.2)

The conditioned medium, which contained 10,000 units EDF, was concentrated and analyzed using Western blot analysis. From



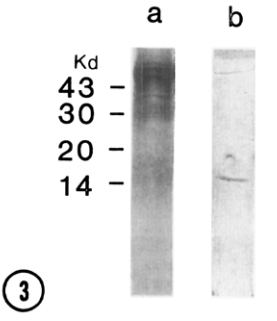


Fig. 2. Expression of EDF subunit mRNA in early transformants and 0.5 M MTX resistant transformants . Each lane contained 10μg of total RNA. The probe was EDF subunit cDNA. (lane a : early transformants, lane b : 0.5μM MTX resistant transformants, lane c : CHO DHFR⁻ cells).

Fig 3. Western immunoblot. Concentrate of conditioned medium was subjected in electrophoresis and then blotted. a) Commassie blue stain and b) immunoblotting with antibody to EDF. The migration of molecular weight standard is shown at the left side of gel.

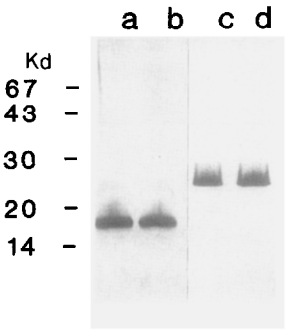


Fig. 4. SDS-Polyacrylamide gel electrophoresis of r-EDF and n-EDF a) n-EDF and b) r-EDF under reducing conditions. c) n-EDF and d) r-EDF under non-reducing conditions.

the result of Western blot analysis, only one band could be detected and its MW was about 15.5kd (Fig.3). EDF subunit precursor contained many di-basic amino acids residues (Lys-Lys, Lys-Arg, Arg-Arg and Arg-Lys), which were putative processing sites. But, the larger band than 15.5kd was not detected in the conditioned medium. EDF was thought to be processed precisely during the secretion process.

r-EDF was purified to homogeneity, and it was confirmed that the biological activity of the r-EDF (differentiation-inducing activity toward mouse Friend leukemia cells) was the same as that of natural-EDF(n-EDF) (data not shown). SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions showed that r-EDF and n-EDF consisted of two identical subunit of the same size (15.5Kd) (Fig.4). In addition, the 18 amino acid N-terminal sequences of r-EDF and n-EDF were identical (N terminal: Gly-Leu-Glu-X-Asp-Gly-Lys-Val-Asn-Ile-X-X-Lys-Lys-Gln-Phe-Phe-Val), and the unknown residues (X) , which could not be identified with the amino acid sequencer.

Thus, CHO cells processed EDF precursor in exactly the same way at five consecutive arginines as THP-1 cells did. CHO cells and THP-1 cells probably have the same processing system, and r-EDF was thought to be folded precisely with a dimeric form structure according to the biological activity of r-EDF.

Recently many lymphokines, including interleukins, are known to have multifunctional activities on different cells in vivo and in vitro. EDF has a differentiation inducing activity to mouse Friend leukemia cells and a FSH releasing activity from cultured pituitary anterior cells in vitro.

We could overproduce r-EDF up to 1μg/ml/3 days using CHO DHFR$^-$/ pSD(X)/EDF system. r-EDF was identical to n-EDF in its structure and its biological activity. The use of r-EDF can allow further studies to determine the respective physiological role in vivo.

Acknowledgement

We are greateful for valuable help and advice to Dr.Tomoko Tuji and Satoshi Takano.

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