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A new human leukemia cell 8.2 kDa differentiation factor: isolation and primary structure

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Abstract A new 8.2 kDa differentiation factor has been purified to homogeneity from the cultural media of human myelogenous HL-60 leukemia cells induced by retinoic acid. cDNA clones encoding this factor were isolated from a cDNA library prepared from HL-60 differentiated cells and their nucleotide sequence has been determined. The deduced amino acid sequence of the differentiation factor molecule consists of 54 amino acid residues. The protein is shown to be glycosylated. It was shown by Northern blot experiments that the level of poly(A)$^+$ RNA with a length of 450 nucleotides was higher in differentiated cells than in non-differentiated cells.

Key words: HL-60 cell; Differentiation factor; Retinoic acid

1. Introduction

Human myelogenous HL-60 leukemia cells provide a unique model system for the analysis of the molecular mechanism of early embryonic determination and differentiation. These cells can be induced to differentiate into either monocyte/macrophage-like cells or into granulocytes by various agents. Thus, all-*trans* retinoic acid mediates granulocytic differentiation [1–3] accompanied by generation of numerous lymphokines [4,5] and growth factors [6].

Earlier we have shown that the new peptide–protein differentiation factors are accumulated in the cultural media of retinoic acid-treated HL-60 cells [7].

We have found a novel 8.2 kDa retinoic acid induced differentiation factor in HL-60 cells. The addition of this factor to HL-60 cells leads to their terminal differentiation in the same way as retinoic acid does.

Studies of this factor and its mechanisms of action can provide new approaches for treatment of certain types of leukemia.

We report here the purification of a 8.2 kDa differentiation factor from the culture medium of HL-60 cells induced by retinoic acid, its cloning and the elucidation of its cDNA primary structure.

2. Materials and methods

2.1. Cell culture

HL-60 cells were obtained from Dr. R.G. Vasilov (Biotechnology Institute, Moscow), and adapted to protein-free RPMI-1640 medium, containing 20 μM ethanolamine, 3 μg/ml ascorbic acid, 500 μm ferrum citrate and microelements. The cells were cultured at 37°C in humidified 5% CO_2 in air and were passaged every 3–4 days at an inoculum size of 2×10^5 cells/ml.

2.2. Cell differentiation

Cell differentiation was evaluated by nitroblue tetrazolium reduction activity (NBT-test) [8]. Viability was assessed by Trypan blue exclusion. One unit of activity of the differentiation factor is defined as the amount necessary to increase the number of NBT-positive HL-60 cells by 10%.

2.3. Purification

The supernatant of 1.5 liter retinoic acid-induced HL-60 cell culture was filtered through a 0.45 μ filter and precipitated with 70% ammonium sulfate. After stirring for 4 hours, the precipitate was resuspended in 1.5 ml 0.1 M Tris-HCl, 0.2 M NaCl pH 7.4 and gel-filtered in the same buffer on Toyopearl HW 55 (Toyosoda, Japan). Fraction 1 (Fig. 1) containing the differentiation activity was dialyzed and futher purified by FPLC on MonoQ HR 5/5.

2.4. Analytical methods

SDS-PAGE was performed according to Laemmli [9]. The proteins were visualized by silver staining [10]. The proteins separated via SDS-PAGE were transferred to an Immobilon P filter (Millipore). The N-terminal sequence was determined by automated Edman degradation using an Applied Biosystem 470A gas-phase sequenator.

RNA was purified according to the acid guanidine–thyocyanate–phenol–chloroform method [11]. Poly(A)$^+$ RNA was isolated [12]. Double-stranded cDNA was synthesized from isolated poly(A)$^+$ RNA using the random cDNA synthesis kit (Amersham) according to the manufacturer's instructions.

Double-stranded cDNA was ligated with *Sma*I-digested plasmid $^+$RNA (5 μg per line) was electrophoresed on 1.2% agarose–formaldehyde gels [12] and transferred to P-labeled cDNA probes at 42°C overnight in the presence of formamide.

DNA manipulations were carried out by standard methods [12].

3. Results and discussion

To facilitate the isolation of the new differentiation factor, HL-60 cells were adapted to a protein-free culture medium.

The differentiation effect of retinoic acid was shown to be much the same in both the adapted HL-60 cells and those cultured with 10% fetal bovine serum.

Isolation of the individual 8.2 kDa factor was done by precipitation of the retinoic acid-induced HL-60 cell cultural media with a 70% saturation of ammonium sulfate followed by gel-

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The nucleotide sequence here has been deposited in the EMBL Nucleotide Sequence Database under accession number X79563 (*H. sapiens* 8.2 kDa differentiation factor mRNA).

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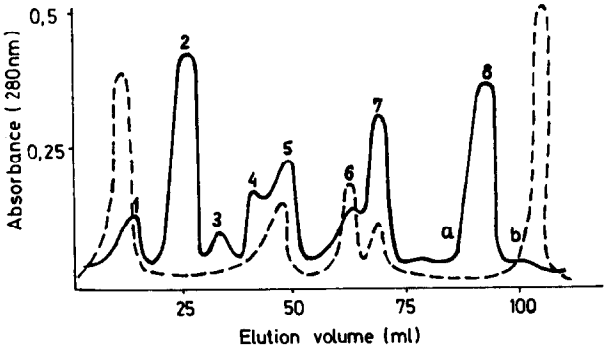

Fig. 1. Gel-filtration on Toyopearl HW55. Solid line, absorbance at 280 nm; dashed line, differentiation activity.

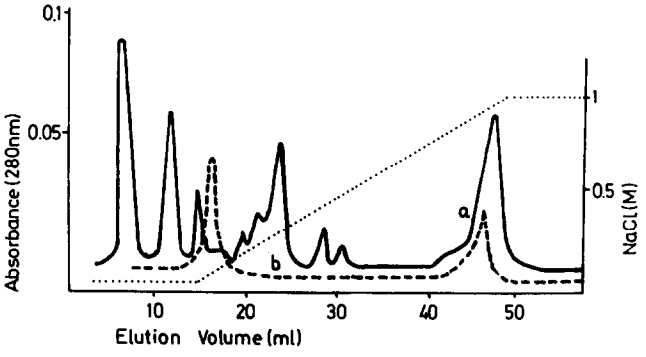

Fig. 2. Purification of 8.2 kDa differentiation factor by ion-exchange chromatography on a Mono Q column: a, absorbance at 280 nm; b, differentiation activity.

filtration on Toyo earl HW55 (Fig. 1). Fraction 1 with the maximum differentiation activity was dialyzed and purified by Mono Q HR5/5 anion ex hange chromatography

The differentiation activity of the protein correlated with its ability to decrease the proliferation level determined by [^{3}H]thymidine incorporation into the cells [14] (Table 2).

Our attempts to digest this factor with trypsin, chemotrypsin and lysilendopeptidase were unsuccesful. This is apparently connected with the high level of glycosylation of this factor as has been shown by carbohydrate analysis (data not shown).

Oligonucleotide probes corresponding to the N-terminal sequence were synthesized (I. GCCGGAATCATGGCCAG, II. GCTGG^G_TATT ATGGC^G_TAG, III. CT^G_TGCCATGA-T^G_TCCGGC). A random hexanucleotide-primed cDNA library prepared from HL-60 differentiated cells was hybrydized with these oligonucleotides. Two hybridization-positive clones (H7 and E8) were isolated from 3×10^5 independent clones analyzed and their inserts were used for sequence analysis. The H7 clone contained full-length cDNA coding the 8.2 kDa differentiation factor.

Fig. 4 shows the nucleotide sequence of the 8.2 kDa differentiation factor cDNA. There are two potential initiation codons (positions 61–63 and 160–162). According to Kozak [15] and Cavener [16] the translation initiation sites of vertebrates include some general features: a strong preferences for purines at the −3 position, a periodical increase in the frequency of G at positions −9, −6, and −3 and a preference for A or C at positions −5, −4, −2 and −1. It was shown that the initiation of translation starts from the first AUG codon when the 5′-noncoding region contains more than 12 nucleotides [15]. More likely, the first AUG codon (61–63) initiates protein synthesis. Codon AAA (371–373), corresponding to the Lys residue, is followed by the termination codon (374–376). The 8.2 kDa differentiation factor cDNA contains two AATAAA sequences (positions 350–355 and 346–351) that can be used as polyadenylation sites.

The factor's N-terminal Ala was found at a distance of 42 amino acids downstream from the suggested initiatory Met. We suggest that proteolytic processing takes place after factor synthesis.

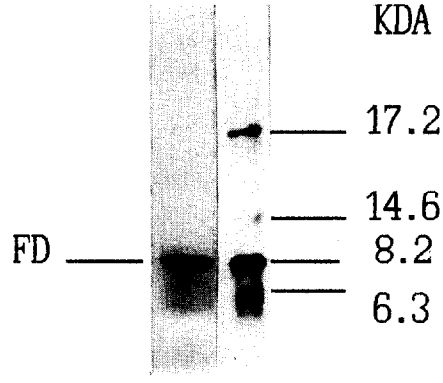

Fig. 3. SDS-electrophoresis of the fraction with maximal differentiation activity after ion-exchange chromatography on the MonoQ column. left, purified differentiation factor; right, molecular mass markers.

Table 1
Purification of the 8.2 kDa differentiation factor

Step	Activity (mmol)	Proteins (mg)	Specific activity (mmol/mg)	Purification (-fold)	Yield %
Culture medium	300	600	0.5	1	100
Precipitation by $(NH_4)_2SO_4$	150	200	0.75	1.5	50
Toyopearl HW 55	75	0.5	150	300	10
Mono Q	4.8	0.024	2000	4000	0.5

Table 2
Differentiation activity of the 8.2 kDa protein factor in the NBT-test and its ability to supress proliferation measured by [^{3}H]thymidine incorporation

Testing substance	Incorporation of [^{3}H]thymidine into HL-60 cells (%)	Increase of NBT-positive cells (%)
Control (non-stimulated cells)	100	–
8.2 kDa differentiation factor (10^{-8} M)	66	32
Retinoic acid (10^{-6} M)	30	60

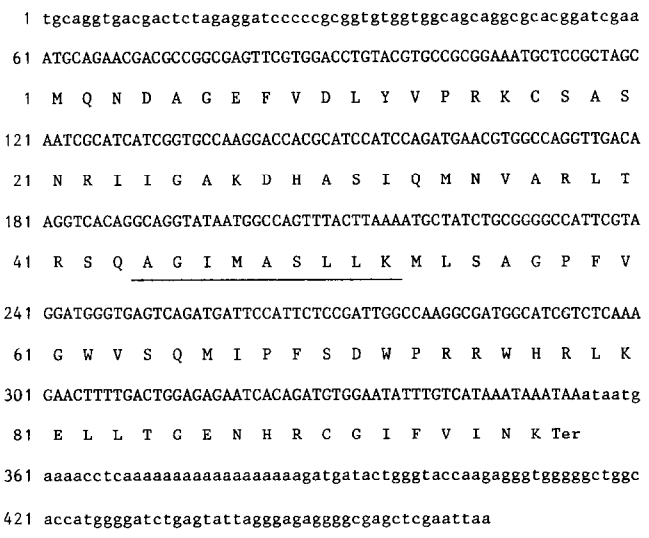

Fig. 4. Nucleotide sequence of 8.2 kDa differentiation factor cDNA and the deduced amino acid sequence. The underlined region indicates the position of the N-terminal sequence.

Thus the 8.2 kDa differentiation factor molecule consists of 54 amino acid residues with a calculated molecular mass of 6200 Da. Most likely, the other part of the protein molecule is carbohydrate moisture.

The level of poly(A)$^+$ RNA with a length of 450 nucleotides has been shown by Northern blot experiments to be higher in differentiated cells in comparision with non-differentiated cells (data not shown). Poly(A)$^+$ RNA from differentiated cells also contains a small amount of 1200 nucleotides poly(A)$^+$ RNA which indicating a positive hybridization signal. We assume that it is a high molecular mass precursor.

Experiments on the expression of this factor are continuing and we believe it can be useful for treatment of certain types of leukemia.

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