TWO SEPARATE DIFFERENTIATION INDUCING PROTEINS FOR HUMAN MYELOID LEUKEMIA CELLS AND THEIR ISOLATION FROM NORMAL LYMPHOCYTES

MOHSEN ABOLHASSANI, W. MICHAEL RILEY, ERIC FELDMAN, Z. ARLIN AND J.W. CHIAO.

Department of Medicine, New York Medical College, Valhalla, NY 10595.

ABSTRACT

Conditioned medium from mitogen stimulated normal peripheral blood lymphocytes (PBL) has been demonstrated to contain a maturation inducer activity mediating the differentiation of human myeloid leukemia cells to monocytes and macrophages. The maturation inducer activity was isolated by salt precipitation, Sepharose CL-6B ion exchange and affinity chromatographies and electrophoresis. Two separate activities with M.W. ranges of 52-56 and 32-35 kDa capable of mediating the terminal differentiation of leukemic HL-60 promyelocytes to monocytes and macrophages were detected. The higher molecular weight species was determined to be a 54 kDa single polypeptide and was found to be distinct from IL-3 and IL-6 by ELISA and differentiation blocking assay. The inducing activity of the 32-35 kDa material was largely neutralized after treatment with anti-IL-3, but not with other anibodies. Employing the immunofluorescent antibody technique, the 54 kDa protein was detected on the surface membranes of PBL. The proportions and number of maturation inducer bearing lymphocytes in patients with acute myelogenous leukemia (0.4% and 35/mm^3, respectively) were significantly lower than that of healthy donors (7.9% and 178/mm^3) The role of these physiological factors in leukemia cell differentiation is discussed.

INTRODUCTION

Specific human physiological factors capable of inducing the differentiation of human myeloid leukemia cells have been described since the late 70's. Initially, an activity from activated normal human T cells was found to mediate the differentiation of human myeloid leukemia cells to mature monocytes and macrophages (1). It mediates a growth suppression of the leukemia cells in parallelwith an induction of terminally differentiated monocytic cells. This activity induces in vitro differentiation of leukemia cells from patients and from myeloid cell lines such as HL-60 and U937 cells (1-3). It does not stimulate the growth of colony forming cells and has been shown to be distinct from colony stimulating factors, interferons and tumor necrosis factor, etc. (1,4).

Similar mouse differentiation inducing factors, effective for the differentiation of mouse myeloid leukemia cells, have been described (5,6). Recently, one of these activities was cloned (7). The nature and quantity of human maturation inducing activities from normal lymphocytes have not been clearly elucidated. In this paper, we have attempted to determine the number of maturation inducing factors and to isolate them from activated normal lymphocytes. The isolation of two such factors and their comparison with other hematopoietically active factors are presented.

MATERIALS AND METHODS

Isolation of Maturation Inducers. Serum free normal lymphocyte conditioned medium (CM) was used to isolate maturation inducing activity. Essentially, peripheral blood lymphocytes (PBL) isolated from normal blood were cultured at 1 x 10^6 cell/ml in RPMI 1640 with 0.1% bovine serum albumin and 0.8% phytohemagglutinin (4). The culture medium from 3-4 day cultures was collected as CM.

CM was concentrated using a Millipore Pellicon Cassette system with a PTGC membrane (MW cut-off = 10 kDa). Concentrated CM was precipitated with 50 % ammonium sulfate and then with 70% ammonium sulfate. The 70% precipitate was dialyzed against 0.01 M Tris buffer, pH 8.0, and then fractionated on a DEAE-Sepharose CL-6B ion exchange column equilibrated with the same buffer. A stepwise NaCl gradient was used. The 70% precipitate was also dialyzed against 0.1 M borate buffer, pH 8.5, and passed over an antibody affintiy column with reactigel 6X (Pierce Chemical Co., Rockford, IL) coupled with the monoclonal rat anti-maturation inducer antibody (3). The maturation inducer was eluted with 1M NaCl. SDS-PAGE was carried out with a 10% acrylamide gel as described (8). After electrophoresis, proteins were extracted from the gel by eluting ground excised areas of an unstained gel with a 1% SDS in saline solution.

Assays. The liquid culture assay with human leukemia cell line HL-60 promyelocytes (4) and ELISA assays were used to analyze the maturation inducer activity. HL-60 cells were cultured at 0.25 x 10^6 cells/ml of RPMI medium with 15% FCS and various concentrations of the maturation inducer preparations. Cultures supplemented with appropriate control medium were carried out with each analysis. The development of monocytes/macrophages, as determined by the acquisition of membrance complement receptors, phagocytic function, α-Naphthyl Acetate Esterase enzyme activity, monocyte membrane antigen CD36, morphological characteristics and cell cycle analysis, were assessed as described (3,4).

The proteins were analyzed with a mouse monoclonal anti-human IL-3 antibody (Genzyme, Boston, MA), a rabbit anti-human IL-6 antibody (Genzyme) and a monoclonal rat anti-human maturation inducer IgG (3) by the ELISA procedure.

0037-9727/90/1952-0288$2.00/0

The maturation inducing preparations were also incubated with these antibodies for 1 hr at 37°C and overnight at 4°C. These mixtures or the antibodies alone, which were used as controls, were then analyzed in the HL-60 cell differentiation assay.

Maturation Inducer on Lymphocytes. Peripheral blood from healthy donors and patients with acute myelogenous leukemia (AML) was reacted with the monoclonal rat anti-maturation inducer IgG. All AML patients were untreated at the time of the study. Their white cell count ranged from 1.65 x 10^3 to 3.3 x 10^3/mm^3. The indirect fluorescent antibody technique was performed on an Ortho 50H cytofluorograph, allowing for grating to examine lymphocyte, monocyte and granulocyte regions. A fluorescent goat anti-rat IgG and a $F(ab')_2$ reagent were used as the second antibody. An IgG preparation from a nonimmunized rat was used instead of the anti-maturation inducer antibody as a background control.

RESULTS

Isolation of Maturation Inducers. Maturation Inducer activity was purified from the CM of mitogen stimulated normal PBL and the activity was assayed by the differentiation induction of human HL-60 myeloid cells. The activity was detected in the 50-70% ammonium sulphate precipitate and in the 0.4 M NaCl eluate from a CL-6B ion exchange column and after electrophoresis on, and extraction from a nonreducing SDS gel. The maturation inducing activity was detected in two molecular weight ranges of 52-56 kDa and 32-35 kDa where a major band was observed at 34 kDa. The higher MW fraction reacted with a rat IgG monoclonal antibody against the maturation inducer activity and affinity chromatography was employed for further isolation.

Purified maturation inducing activity was identified in the SDS gel eluate within the MW range of 52-56 kDa, as determined by both the bioassay and ELISA. Figure 1 shows that a single band was detected in this range and it possessed an apparent MW of 54 kDa. SDS-PAGE of the 54 kDa protein under reducing conditions revealed only one band, indicating this is a single polypeptide chain with no subunits.

Differentiation Activity. Addition of the purified 54 kDa protein or the 34 kDa preparation to HL-60 cells produced gradual changes in the cellular characteristics, indicative of differentiation into monocytes and macrophages. As little as 10^{-7} µg/ml of the 54 kDa protein induced an observable development of monocytic cells with optimal activity achieved at 10^{-4} µg/ml. Maturation induction was observed for the 34 kDa preparation at does as low as 2 x 10^{-1} µg/ml with an optimum dose of 1.0 µg/ml. Table 1 presents the results of cell characteristic analysis at optimal maturation induction conditions. Differentiated cells acquired phagocytic capacity and membrane complement receptors which increased with longer induction periods. The differentiated cell types, determined to be monocytes and macrophages by morphology with the development of monocytic cell enzyme α-naphthyl acetate esterase activity, showed an increase in parallel with a decrease in the promyelocytic population (Table 1). The differentiated cells also acquired the monocyte membrane antigen CD36, with approximately 50% of treated cells testing positive as detected by immunofluorescence on day 5 compared to < 1% of untreated HL-60 cells.

Along with the development of mature cells, the proliferation of the cell cultures became static, leading to a termination of these differentiated cells. The number of viable cells in the differentiated cell cultures decreased as well as replicating cells as determined by cell cycle analysis. Approximately 83% of treated cells were in G_1 phase and 17% in the proliferating phases SG_2M as compared to untreated HL-60 cells which had 60% cells in G_1 and 40% SG_2M cells.

Table 1 also shows that the 34 kDa activity was blocked by incubation with an anti-IL-3 antibody but not with either anti-IL-6 or the anti-maturation inducer antibodies. By contrast, the 54 kDa protein lost its differentiation activity when trated with the anti-maturation inducer antibody but not with the antibodies against the interleukins. These observations were confirmed with the ELISA procedure. The 34 kDa preparation reacted only with the anti-IL-3 antibody, while the 54 kDa protein reacted with the anti-maturation inducer antibody alone.

Maturation Inducer on Lymphocytes. The presence of the 54 kDa maturation inducer on lymphocytes was analyzed by the indirect fluorescent antibody technique employing the rat IgG monoclonal antibody against the human maturation inducer. Differential gating on an Ortho 50H cytofluorograph was used to analyze lymphocytes, monocytes and granulocytes. Only lymphocytes showed positive fluorescence compared to the control nonimmune rat IgG. Table 2 shows the detection of these lympho-

Table II. Presence of Maturation Inducer-Bearing Lymphocytes in Normal Persons and Acute Myelogenous Leukemia Patients

Donor	Maturation inducer-positive lymphocytes			
	%		Absolute Number/ mm^3	
	Mean ± SD	Range	Mean ± SD	Range
Normal (9)[a]	7.9 ± 7.4	1–20.7	178 ± 165	76–304
Leukemic patients (6)	0.4 ± 0.5	0–1.2	35.3 ± 52.5	0–135
	$P < 0.001$[b]		$P < 0.01$[b]	

[a] Numbers in parentheses, patients.
[b] Probability value for comparing acute myelogenous leukemia patients with normal donors.

Table I. Differentiation Induction of Human HL-60 Cells with Isolated Maturation Inducing Preparations

Culture condition	Inducer quantity (µg/ml)	Cell characteristics (%)				Differentiation blocking by		
		Complement receptor	Phagocytosis	Promyelocytes	Monocytes/ macrophages[a]	Anti-IL-3	Anti-IL-6	Anti-54 kDa MI
HL-60	0	2	3	98	0	–	–	–
HL-60 + 54 kDa MI	10^{-4}	45	37	69	31	–	–	
HL-60 + 34 kDa MI	1.0	54	17	68	32	+	–	–

[a] Percentage of monocytes/macrophages are average values of morphologic assessment and presence of α-naphthyl acetate esterase activity.

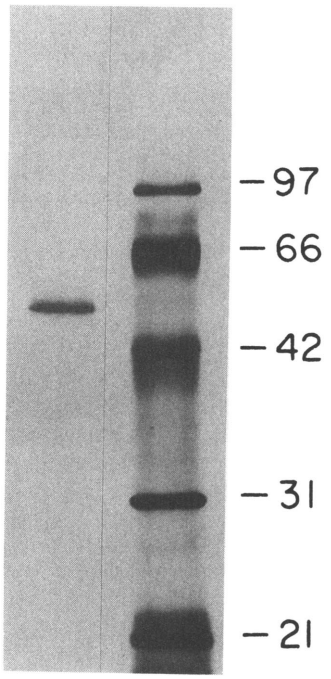

Figure 1. SDS-PAGE of isolated maturation inducer in the absence of reducing agent. Left lane, purified maturation inducer protein; right lane, molecular weight markers × kDa. The gels were silver stained

cytes from healthy donors and patients with AML. For the leukemia patients, approximately 50% of the AML patients showed an absence of maturation inducer bearing lymphocytes after analyzing their lymphoid and myeloid cell populations. The average percent of positive cells in patients with AML (0.4%) and their average absolute number of positive cells (35.3/mm^3) were significantly lower than those of healthy donors (7.9% and 178/mm^3).

DISCUSSION

Using CM from activated normal human lymphocytes, the presence of differentiation inducing activity for myeloid leukemic cells was isolated. Two separate activities were identified. One activity was purified to apparent homogenity and appeared to be a single polypeptide protein with an apparent MW of 54 kDa and an optimum activity of approximately 2 pM. The molecular weight of this maturation inducer agrees with that of a maturation inducer previously isolated from a human T cell line (3). The second activity, which was only partially purified, was shown to be in the MW range of 32-35 kDa with optimum activity at about 29 nM. The *in vitro* activity ranges of these two factors corresponds with the humoral ranges of other known growth factors.

The 32-35 kDa preparation did not react with the antibody against the 54 kDa maturation inducer, indicating that these two activities may represent two different molecules, although this remains to be confirmed. Since anti-IL-3 antibody effectively blocked the differentiation induction of the 34 kDa species, the sharing of antigenicity with IL-3 is suggested. This is in line with other observations on the role of IL-3 in myeloid cell growth and differentiation (9,10).

The finding of the presence of maturation inducer on peripheral blood lymphocytes from healthy persons reaffirms the earlier finding that lymphocytes are involved in its production (1). In contrast to normal individuals, patients with AML were either absent or deficient in the number and percentage of these lymphocytes. These results have indicated a possible relationship of these lymphocyte subpopulations and the maturation inducer molecules to the pathogenesis of leukemia diseases.

The finding of two separate maturation inducing activities in lymphocyte conditioned medium is similar to the original observation of mouse differentiation factors. Yamamoto et al (11) described that mitogen stimulated spleen cells produce two differentiation factors with MW ranges of 40-50 kDa, produced by lymphocytes, and 22-25 kDa, produced by macrophages. Recently, one of the murine differentiation factors has been cloned and shown to be nearly identical to the murine leukemia inhibitory factor (7). The nature of the human leukemia inhibitory factor and its relationship to the 54 kDa species of human maturation inducer has not been fully elucidated. Our finding that the 54 kDa maturation inducer may be distinct from IL-6 is supported by others (12) who determined that IL-6 possesses differentiation activity for certain myeloid precursors in soft agar gel, but does not effect the growth and diff- of HL-60 cells. Physiological factors such as IL-6, IL-3 and the maturation inducer, etc., may therefore represent a family of factors mediating monocytic cell development.

Acknowledgement: This research was supported in part by NIH grants CA35999 and 36040. The excellent technical assistance of Mr. John Rossi is greatly appreciated.

Correspondence and Reprint Requests to:
Dr. J.W. Chiao, 207 Vosburgh Pavillion, New York Medical College, Valhalla, NY 10595, (914)-993-4199.

REFERENCES

1. Chiao JW, Freitag WF, Steinmetz JC, Andreef M. Changes of cellular markers during differentiation of HL-60 promyelocytes to macrophages as induced by T lymphocyte conditioned medium. Leukemia Res 5:477-489, 1981.
2. Chiao JW, ANdreef M Freitag WF, Arlin Z. Induction of in vitro proliferation and maturation of human aneuploid myelogenous leukemic cells. J Exp Med 155:1357-1369, 1982.
3. Chiao JW, Abolhassani M, Leung K, Heil M. Mechanism of regulating human leukemia cell growth and differentiation by a lymphokine. Biochem Biophys Res Comm 161: 583-588, 1989.
4. Leung K, Chiao JW. Monoclonal antibodies neutralizing the maturation inducer activities for myeloid leukemia cells. Fed Proc 46:785-786, 1987.
5. Ichikawa Y. Differentiation of a cell line of myeloid leukemia. J Cell Physiol 74:223-234, 1969.
6. Sachs L. Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. Nature (London) 274:535-539, 1978.
7. Metcalf D, Nicola NA. Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. J Cell Physiol 116:198-206, 1983.
8. LaemmliUUK. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. Natur (London) 227:265-275, 1970.
9. Keller JR, Ihle JN. Unique pathway of IL-3-driven hemopoietic differentiation. J Immun 143:4025-4033, 1989.
10. Ottmann OG, Abboud M, Welte K, Souza LM, Pelus LM. Stimulation of human hematopoietic progenitor cell proliferation and differentiation by recombinant human interleukin 3. Comparison and interactions with recombinant

human granulocyte- macrophage and granulocyte colony-stimulating factors. Exp Hematol 17: 191-197, 1989.
11. Yamamoto Y, Tomida M, Hozumi M. Production by mouse spleen cells of factors stimulating differentiation of mouse myeloid leukemic cells that differ from the colony stimulating factor. Cancer Res 40:4804-4809, 1980.
12. Onozaki K, Akiyama Y, Okana A, Hirano T, Kishimoto T, Hashimoto T, Yoshizawa K, Taniyama T. Synergitic regulatory effects of interleukin 6 and interleukin 1 on the growth and differetniation of human and mouse myeloid leukemia cell lines. Cancer Res 49: 3602-3607, 1989.

Received May 31, 1990
Accepted September 7, 1990