The EMBO Journal Vol.1 No.11 pp.1343-1346, 1982

Autoinduction of differentiation in myeloid leukemic cells: restoration of normal coupling between growth and differentiation in leukemic cells that constitutively produce their own growth-inducing protein

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Communicated by Leo Sachs
Received on 6 September 1982

Growth and differentiation of normal myeloid haematopoietic cells are regulated by a family of macrophage- and granulocyte-inducing (MGI) proteins. Some of these proteins (MGI-1) induce cell growth and others (MGI-2) induce cell differentiation. Addition of MGI-1 to normal myeloid cells induces growth and also induces the endogenous production of MGI-2. This induction of differentiation-inducing protein by growth-inducing protein then ensures the coupling between growth and differentiation found in normal cells. There are myeloid leukemic cells that constitutively produce their own MGI-1, but the cells do not differentiate in culture medium containing horse or calf serum. By removing serum from the medium, or in medium with mouse or rat serum, these leukemic cells are induced to differentiate to mature cells, which like normal mature cells, then no longer multiply. Leukemic cells with constitutive production of MGI-1 continuously cultured in serum-free medium with transferrin were also induced to differentiate by removing transferrin. This induction of differentiation was in all these cases associated with the endogenous production of MGI-2 by the cells. The results indicate that changes in specific constituents of the culture medium can result in autoinduction of differentiation in these leukemic cells due to restoration of the induction of MGI-2 by MGI-1, which then restores the normal coupling of growth and differentiation.

Key words: constitutive gene expression/coupling of growth and differentiation/differentiation-inducing protein/growth-inducing protein/leukemic myeloid cells

Introduction

There are different macrophage- and granulocyte-inducing (MGI) proteins that induce the growth and differentiation of normal myeloid haematopoietic cells. Some of these proteins, which we now call MGI-1, induce cell growth (multiplication), and other proteins, which we now call MGI-2, induce cell differentiation (reviewed in Sachs, 1978, 1980, 1982a, 1982b; Lotem and Sachs, 1982). We have also shown that in normal myeloid cells the growth-inducing protein MGI-1 can induce production of the differentiation-inducing protein MGI-2, and this thus provides an effective mechanism for coupling growth and differentiation in normal cells (Sachs, 1980, 1982a; Liebermann *et al.*, 1982; Lotem and Sachs, 1982). Induction of growth in normal cells requires addition of MGI-1 from an external source and the regulation of growth and differentiation in these cells can be studied in culture (reviewed in Sachs, 1974, 1978, 1980, 1982a). MGI-1 has also previously been referred to as mashran gm (Ichikawa *et al.*, 1967), colony-stimulating factor (CSF) (Metcalf, 1969), colony-stimulating activity (CSA) (Austin *et al.*, 1971), and MGI (Landau and Sachs, 1971).

There are different types of myeloid leukemic cells. Some no longer require MGI-1 for growth or require an external source of MGI-1. However, in both these types of cells, MGI-1 did not induce MGI-2, so that growth and differentiation have been uncoupled (Sachs, 1978, 1980, 1982a; Liebermann *et al.*, 1982; Lotem and Sachs, 1982). There are also other myeloid leukemic cells that constitutively produce their own MGI-1 (Paran *et al.*, 1968; Ralph *et al.*, 1978). However, unlike in normal cells, the presence of MGI-1 did not result in differentiation in these leukemic cells in the culture medium used.

The present experiments were carried out to determine whether the induction of MGI-2 by MGI-1 can be restored in these leukemic cells and whether this then also restores the normal coupling of growth and differentiation. The results indicate that changing specific constituents in the culture medium in leukemic cells that constitutively produce MGI-1 can restore the induction of MGI-2 by MGI-1. This then results in an autoinduction of differentiation in these leukemic cells by restoring the normal coupling between growth and differentiation.

Results

Autoinduction of differentiation in serum-free medium

Six clones of mouse myeloid leukemic cells were used in the present experiments. One of these clones, WEHI-3B, constitutively produced MGI-1, whereas the other five clones (1,5,9,10, and 11) did not produce MGI-1 and no longer required MGI-1 for growth. The transfer of these clones from culture medium with horse serum to serum-free medium resulted in induction of differentiation in WEHI-3B cells (Table I). This included induction of the differentiation-associated properties of C3 and Fc rosette formation, lysozyme synthesis, and the formation of mature cells. These mature cells, like normal mature cells, no longer multiplied. Removal of serum thus induced autoinduction of differentiation in these leukemic cells. There was also, in the serum-free medium, induction of differentiation without the formation

Table I. Autoinduction of differentiation in serum-free medium

Clone	Horse serum	Cells with rosettes (%)		Lysozyme (μg equivalent/ 5×10^6 cells)	Mature cells (%)
		Fc	C3		
WEHI-3B	+	10 ± 1.5	8 ± 1.2	0.4 ± 0.1	0
	−	25 ± 4	21 ± 3	1.9 ± 0.3	15 ± 2.4
9	+	0.4 ± 0.2	0.5 ± 0.2	0	0
	−	2.8 ± 0.4	4.3 ± 0.5	0.8 ± 0.2	0
1,5,10,11	+	0.7 ± 0.2	0.5 ± 0.2	0	0
	−	0.4 ± 0.1	0.6 ± 0.2	0	0

Cells grown in serum-containing medium (EM and 10% horse serum) were washed and transferred to serum-containing or serum-free medium (EM and 5 μg/ml transferrin). The differentiation-associated properties were measured after 4 days.

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of mature cells in clone 9, but no induction of differentiation in any of the other clones (Table I).

Blocking of autoinduction of differentiation by different sera

Experiments with sera from different species have shown that calf serum, like horse serum, inhibited autoinduction of differentiation in WEHI-3B cells. The use of 1% serum gave a similar inhibition to 10% serum. However, WEHI-3B cells cultured in medium with 1% rat or mouse serum were autoinduced for differentiation like cells cultured in serum-free medium (Table II). Similar results were obtained with syngeneic (Balb/c) and allogeneic (SL and ICR) mouse sera. This shows that autoinduction of differentiation can occur in these cells in serum from the same species as the leukemic cells, mouse, and even in rat serum, but that it is blocked in horse and calf serum. Clone 9 also showed induction of differentiation in mouse and rat but not in calf or horse serum, and the other clones (1,5,10, and 11) showed no induction of differentiation in any of these sera.

Regulation of autoinduction by transferrin

Since WEHI-3B and clone 9 cells were induced to differentiate (Table I) in serum-free EM medium containing transferrin (see Materials and methods), it was of interest to determine the role of transferrin in this induction. Removal of transferrin from this medium resulted in the death of clone 9 cells within 1–2 days, as previously found with clones 1,5,10, and 11 (Symonds and Sachs, 1982a), whereas the WEHI-3B cells remained viable and showed an even stronger induction of differentiation. After 4 days there were 40 ± 6% mature cells without transferrin compared to 15 ± 2% mature cells with transferrin. There was no further increase in the total number of cells after 4 days and 100% of the cells were attached mature macrophages by day 8.

It has previously been shown (Symonds and Sachs, 1982a) that clones 1,5,10, and 11 can be continuously cultured in a serum-free medium consisting of IMDM (see Materials and methods) and transferrin. The change from medium containing horse serum to this serum-free medium gave induction of differentiation in WEHI-3B and clone 9 cells, but it was possible in both these clones to select cells that continued to grow (Figure 1) and were no longer induced to differentiate in this medium. The doubling times and saturation densities were similar to those obtained with other myeloid leukemic clones continuously grown in IMDM with transferrin (Symonds and Sachs, 1982a). These cells were continuously cultured in this serum-free medium for >3 months. Removal of transferrin from the medium again resulted in the death of clone 9 cells, whereas in these selected WEHI-3B cells the lack of transferrin induced differentiation. After 4 days the amount of lysozyme increased from 0.3 ± 0.1 to 1.3 ± 0.2 μg equivalent/5 x 10^6 cells, and intermediate stages of morphological differentiation from 4 ± 1% to 13 ± 3%. These results show that autoinduction of differentiation in WEHI-3B cells can be regulated by transferrin.

Table II. Blocking of autoinduction of differentiation by different sera

Clone	Serum	Lysozyme (μg equivalent/5 x 10^6 cells)
WEHI-3B	Horse or calf	0.5 ± 0.2
	Mouse or rat	1.9 ± 0.4
	None	2.2 ± 0.3
9	Horse or calf	0
	Mouse or rat	0.7 ± 0.3
	None	0.8 ± 0.2
1,5,10,11	Horse, calf, mouse, rat, or none	0

Cells were grown for 4 days in EM and 5 μg/ml transferrin with or without 1% of the sera shown and then tested for the synthesis of lysozyme. Similar results were obtained with mouse serum from the strains Balb/c, SL, and ICR.

Induction of the differentiation-inducing protein MGI-2

In order to determine whether the induction of differentiation in WEHI-3B and clone 9 by removal of horse serum, and in the selected WEHI-3B cells by removal of transferrin, was mediated by production of the differentiation-inducing protein MGI-2, culture supernatants and cell extracts were examined for the presence of MGI-2. Neither of these clones showed any detectable MGI-2 activity before induction of differentiation. The results show (Table III), that induction of differentiation in these cells was always associated with the induction of MGI-2 and the amount of MGI-2 induced was associated with the degree of differentiation. MGI-2 activity was already produced by WEHI-3B cells at 12 h after induction, at the time when the induction of C3 rosettes (and Fc rosettes) was detected, and before the induction of lysozyme synthesis which was first detected at 2 days (Figure 2). These data indicate that the induction of differentiation was mediated by the production of MGI-2. WEHI-3B cells constitutively produced the growth-inducing protein MGI-1 before induction of differentiation, and they continued to produce similar amounts of MGI-1 after this induction (Table III).

The MGI-2 activity shown in Figure 2 was all cell-associated up to 2 days and from 3 to 5 days ~50% was found in the culture supernatants. The MGI-2 induced in WEHI-3B and clone 9 was active on WEHI-3B, clone 9 and

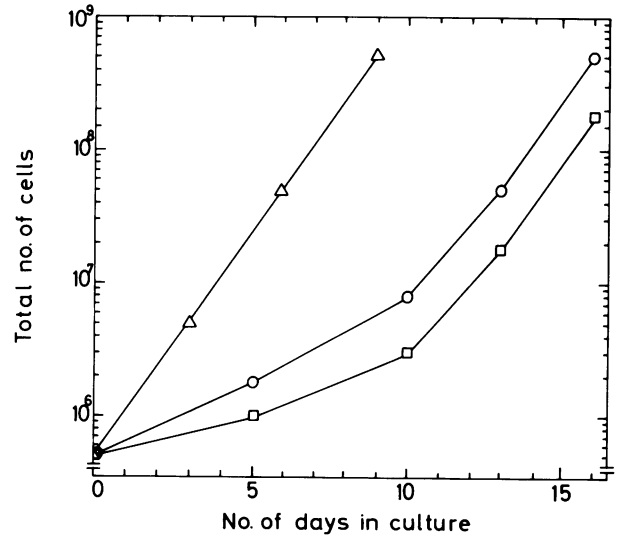

Fig. 1. Continuous growth of leukemic cells in serum-free medium of IMDM and transferrin. Cells cultured in EM with 10% horse serum were washed and seeded at 1 x 10^5 cells/ml in IMDM and 5 μg/ml transferrin. They were subcultured at 3–5 day intervals at the same seeding level. The number of cells was counted at the time of subculture and the total number of cells calculated from the overall increase in cell number. WEHI-3B (□), clone 9 (○), clone 11 (△).

on clone 11 cells in medium with horse serum (Table IV). This MGI-2 therefore induced differentiation in the clone in which it was produced and also in other clones. There is a different clone of myeloid leukemic cells, clone 7-M18 (Lotem and Sachs, 1977), which can be induced to differentiate by insulin (Symonds and Sachs, 1982b) and tumor promoting phorbol esters (Lotem and Sachs, 1979) in medium with horse serum. WEHI-3B, but not clone 9, before induction of differentiation by the procedures described above, contained a differentiating-inducing activity for clone 7-M18 (Lotem and Sachs, in preparation). However, this differentiating-inducing activity did not induce differentiation in medium with horse serum in WEHI-3B, clone 9, or clone 11.

Discussion

The comparison of normal and leukemic myeloid cells has indicated that the origin of myeloid leukemia is due to a change from an induced to a constitutive expression of genes that control cell growth (Sachs, 1978, 1980, 1982a). This change from induced to constitutive gene expression can be produced by the suggested integration of proviral sequences near growth regulatory sites (Liebermann and Sachs, 1979; Liebermann *et al.*, 1980), or by specific chromosome changes that produce differences in the balance of certain genes due to changes in gene dosage (Sachs, 1974; Azumi and Sachs, 1977). Studies with avian leukosis virus have provided direct evidence that proviral sequences can be integrated near growth regulatory genes, and that this can activate these genes (Hayward *et al.*, 1981; Payne *et al.*, 1982). It seems that integration of proviral sequences has to be followed by chromosome changes for the cells to be malignant (Liebermann and Sachs, 1979; Sachs, 1982a). There is one type of leukemic cell in which this change to constitutive gene expression results in constitutive production of the normal growth-inducing protein MGI-1. Induction of growth in normal cells requires addition of MGI-1 from an external source. The constitutive production of MGI-1 thus gives a growth advantage to these leukemic cells.

The present data have shown that myeloid leukemic cells which constitutively produce MGI-1 were induced to differentiate either by removing serum from the culture medium, or by changing the type of serum in the medium. The selection of cells that continuously grew in serum-free medium with transferrin has also shown that differentiation can be induced in these cells by removing transferrin from the culture medium. This induction of differentiation in these cells was always associated with production of the normal myeloid differentiation-inducing protein MGI-2, and the amount of MGI-2 produced was related to the degree of differentiation.

There are clones of myeloid leukemic cells that can be induced to differentiate by MGI-2 without producing MGI-1.

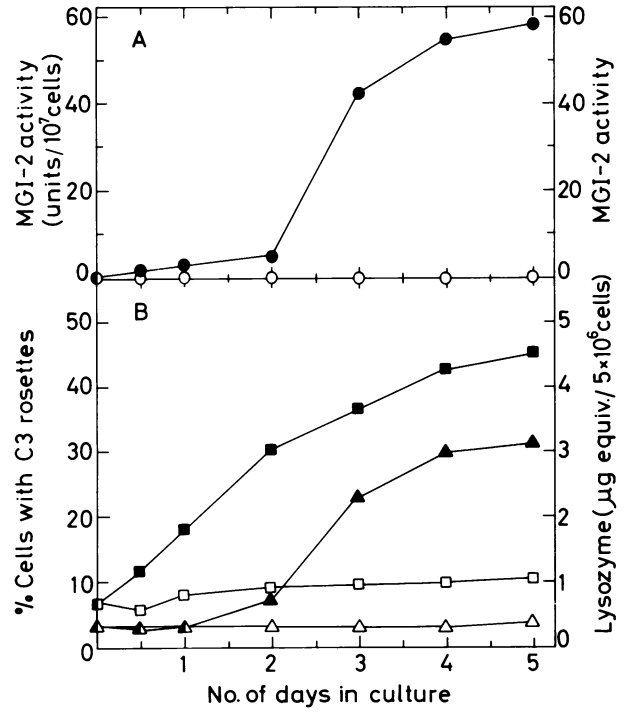

Fig. 2. Induction of MGI-2, C3 rosettes, and lysozyme synthesis during autoinduction of differentiation in WEHI-3B cells in serum-free medium. **(A)** Production of MGI-2 in serum-containing (○) and serum-free (●) medium; **(B)** C3 rosettes in serum-containing (□) and serum-free (■) medium and lysozyme production in serum-containing (△) and serum-free (▲) medium. Cells grown in serum-containing medium (EM and 10% horse serum) were washed and transferred to serum-containing or serum-free medium (EM without transferrin). MGI-2 was assayed on clone 11 in EM and 10% horse serum.

Table III. Autoinduction of differentiation and induction of MGI-2

Clone	A. Effect of horse serum			B. Effect of transferrin		
	Horse serum	MGI activity (units/10^7 cells)		Transferrin	MGI activity (units/10^7 cells)	
		MGI-1	MGI-2		MGI-1	MGI-2
WEHI-3B	+	988 ± 112	0	+	922 ± 127	0
	−	1321 ± 217	58 ± 11	−	1073 ± 131	27 ± 4
9	+	0	0	+	0	0
	−	0	7 ±2	−	C.D.	C.D.
1,5,10,11	+	0	0	+	0	0
	−	0	0	−	C.D.	C.D.

Cells continuously cultured in: **A**, serum-containing medium (EM and 10% horse serum); or **B**, serum-free medium (IMDM and 5 μg/ml transferrin) were tested for production of MGI-1 and MGI-2 with or without horse serum in **A** and with or without transferrin in **B**. MGI-1 and MGI-2 activities from both culture supernatants and cell extracts were assayed 4 days after cell seeding. MGI-2 activity was assayed on clone 11 in EM and 10% horse serum. As with MGI-2 from other sources (Lipton and Sachs, 1981), MGI-2 activity was destroyed by incubation for 2 h with 400 μg/ml crystalline trypsin, or at 70°C for 30 min. C.D.; cell death.

Table IV. MGI-2 produced after induction of differentiation assayed on different clones

MGI-2 assayed on clone:	MGI-2 activity (units/10^7 cells)		
	Induction by:		
	Removal of serum from:		Removal of transferrin from:
	WEHI-3B	9	WEHI-3B
WEHI-3B	50 ± 9	12 ± 3	20 ± 5
9	15 ± 4	5 ± 2	8 ± 2
11	58 ± 11	7 ± 2	27 ± 4

MGI-2 was induced in WEHI-3B and clone 9 cells cultured in serum-containing medium (EM and 10% horse serum) by removing serum and adding 5 μg/ml transferrin; and in WEHI-3B cells cultured in serum-free IMDM medium and transferrin by removing transferrin. MGI-2 activities from both culture supernatants and cell extracts were then assayed 4 days later on WEHI-3B and clones 9 and 11 in EM with 10% horse serum.

Even when these cells require the addition of MGI-1 for growth, MGI-1 does not induce MGI-2 (Lotem and Sachs, 1982). This type of clone appears to be genetically blocked for the induction of MGI-2 by MGI-1. The present results indicate that there also exists another type of leukemic clone that constitutively produces MGI-1 and in which changes in specific constituents of the culture medium can restore the induction of MGI-2 by MGI-1. These leukemic cells are then autoinduced to differentiate. In this type of clone, the ability of MGI-1 to induce MGI-2 can thus be unblocked by changing certain components of the culture medium, so that the cells can then differentiate by restoring this normal coupling mechanism between growth and differentiation. It will be of interest to determine whether such a restoration of the normal coupling mechanism between growth and differentiation can be obtained in erythroleukemic (Friend, 1978; Marks and Rifkind, 1978; Harrison, 1982) and other types of malignant cells. Since myeloid leukemic cells can differentiate *in vivo* (Lotem and Sachs, 1978, 1981) in addition to differentiating in culture, this differentiation of malignant cells by restoring the normal coupling mechanism may be induced under appropriate conditions *in vivo*.

Materials and methods

Cells and cell culture

Mouse myeloid leukemic clones 5, 9, and 11 were derived (Fibach *et al.*, 1973) from a myeloid leukemia in a SL mouse (Ichikawa, 1969); clones 1, 6, 10, and 7-M18 from X-irradiation induced myeloid leukemias in SJL/J mice (Lotem and Sachs, 1974, 1977); and the WEHI-3B cell line from a myelomonocytic leukemia in a Balb/c mouse (Burgess and Metcalf, 1980). The WEHI-3B cell line was obtained from M.Dexter. The leukemic cells were continuously cultured either in Dulbecco's modified Eagle's medium (EM) with a 4-fold concentration of amino acids and vitamins (H21, Grand Island Biological Co., NY) and 10% horse serum (serum-containing medium), or in a serum-free medium of Iscove's modification of Dulbecco's modified Eagle's medium (IMDM, Grand Island Biological Co., NY) and 5 μg/ml purified human transferrin (Behringwerke, Marburg/Lahn) one-third saturated with Fe^{3+} (Iscove and Melchers, 1978). In continuous cultures in these media, WEHI-3B cells were 95% promyelocytes and 5% intermediate stages of myeloid differentiation and all the other clones were myeloblasts to promyelocytes. Cells cultured in serum-containing medium were washed with serum-free EM before transfer to serum-free medium.

All sera were incubated at 56°C for 30 min before use. Fetal calf serum is referred to as calf serum. Mouse and rat sera were obtained from 2–4 month old mice (Balb/c, SL, and ICR) and Sprague Dawley rats as described (Symonds and Sachs, 1982b). For the experiments, cells were seeded at various densities to give 0.7–1 x 10^6 cells/ml at the time of harvest.

Assays for MGI-1 and MGI-2 activity, rosettes, lysozyme, and cell morphology

MGI-1 activity was assayed by seeding 5 x 10^4 nucleated bone marrow cells in soft agar as described (Pluznik and Sachs, 1965; Lotem *et al.*, 1980) in EM with 20% horse serum and the number of macrophage and granulocyte colonies with >50 cells was counted 7 days after seeding. One unit of MGI-1 activity is defined as the amount inducing the formation of one colony per 5 x 10^4 nucleated bone marrow cells. Unless otherwise stated, MGI-2 activity was assayed by measuring the induction of lysozyme synthesis in clone 11 myeloid leukemic cells in EM with 10% horse serum (Krystosek and Sachs, 1976). One unit of MGI-2 activity is defined as the amount inducing 1 μg lysozyme equivalent per 5 x 10^6 leukemic cells after 4 days. Supernatants from cell cultures, and cell extracts prepared by two cycles of freezing and thawing (Falk and Sachs, 1980), were assayed for MGI-1 and MGI-2 activity and the combined activities from the supernatants and cell extracts are expressed as the total number of units produced by 10^7 cells (Liebermann *et al.*, 1982; Lotem and Sachs, 1982). Rosette formation with sheep erythrocytes coated with antibody (Fc rosettes) or antibody and complement (C3 rosettes) were assayed as previously (Lotem and Sachs, 1974); lysozyme was measured by a turbidometric assay (Krystosek and Sachs, 1976). The presence of serum did not interfere with the lysozyme assay. Morphological differentiation was determined on May-Grunwald-Giemsa stained cytocentrifuge smears. Mature cells in the present experiments were macrophages, as determined by their attachment and spreading on the surface of the Petri dish, an eccentric nucleus, a high cytoplasm/nucleus ratio, and a less basophilic cytoplasm than in immature cells.

Acknowledgements

We are indebted to Mrs.R.Kama for skilful technical assistance. This research was supported by a grant from the Hermann and Lilly Schilling Foundation.

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