Haematology and Blood Transfusion Vol. 29
Modern Trends in Human Leukemia VI
Edited by Neth, Gallo, Greaves, Janka

Normal Regulators of Growth and Differentiation and the Reversal of Malignancy in Leukemia

L. Sachs[1]

A. Cloning and Clonal Differentiation of Normal Hematopoietic Cells in Culture

The cloning and clonal differentiation of normal hematopoietic cells in culture made it possible to study the controls that regulate growth (multiplication) and differentiation of different hematopoietic cell types; see [63–68]. We first showed [17, 59], as was then confirmed by others [4], that normal mouse myeloid precursor cells cultured with a feeder layer of other cell types can form clones of granulocytes and macrophages in culture. We also found that the formation of these clones is due to secretion by cells of the feeder layer of specific inducers that induce the formation of clones and the differentiation of cells in these clones to macrophages or granulocytes in mice [26, 59, 60] and in humans [57]. After we first detected their presence in culture supernatants [26, 60], these protein inducers have been referred to by a number of names and I shall use the name macrophage and granulocyte inducers (MGI) (Table 1). These proteins can be produced and secreted by various normal and malignant cells in culture and in vivo [63]. Their production can be induced by a variety of compounds [10, 12, 41, 80] and some cells produce these proteins constitutively [1, 26, 31, 34, 71]. MGI are a family of proteins that exist in a number of molecular forms that have different biologic activities. This cell culture approach has led to the cloning and isolation of growth factors for all the different types of hematopoietic cells, including different types of lymphocytes.

B. Normal Growth- and Differentiation-Inducing Proteins

The family of MGI proteins include some proteins that induce cell growth (multiplication) and others that induce differentiation. Those that induce growth, which are also required for normal cell viability, we now call MGI-1. These include proteins that induce the formation of macrophage clones (MGI-1M) [26, 48, 71], granulocyte clones (MGI-1G) [26, 48, 54], or both types of clones (MGI-1GM) [6, 31, 34]. MGI-1 has previously been referred to as mashran gm [27], colony-stimulating factor (CSF) [51], colony-stimulating activity (CSA) [1], and MGI [31] (Table 1). The existence of an antibody that does not react with all forms of MGI-1M or MGI-1G has shown that there can be different antigenic sites on molecules that belong to the same form of MGI-1 [47, 48]. The other main type of MGI, which we now call MGI-2 [33, 48, 66], induces the differentiation of myeloid precursor cells, either leukemic [14] or normal [33, 66], without inducing colony formation. This differentiation-inducing protein [13, 14] has also been referred to as MGI [14], D factor [49, 82], and GM-DF [5]. It has been suggested that there are different forms of MGI-2 for differentiation to macrophages or granulocytes [33]. The regulation of MGI-1 and MGI-2 appears to be

1 Department of Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Table 1. In vitro cloning and clonal differentiation of normal hematopoietic cells

Cloning and differentiation in liquid medium (mast cells and granulocytes) [17]
Cloning and differentiation in agar (macrophages) [59]
Inducer for cloning and differentiation secreted by cells [59]
Inducer in conditioned medium from cells (for marcrophages and granulocytes) [26, 60]
Different inducer for macrophage and granulocyte clones [26]
Cloning and differentiation of macrophages and granulocytes in methylcellulose [26]
Confirmation of cloning and differentiation in agar [4]
Production of inducer for cloning by some leukemic cells [56]
Cloning and differentiation of human cells [57, 58]
Protein inducer of differentiation that does not induce cloning [13]
Terminology used for proteins that induce cloning and differentiation of normal macrophages and granulocytes
Mashran gm [27]
Colony-stimulating factor (CSF) [51]
Colony-stimulating activity (CSA) [1]
Macrophage and granulocyte inducers (MGI) [31]
MGI-1 (=mashran gm, CSF, CSA) for cloning; MGI-2 (=D factor, DF) for differentiation [33, 44, 48, 66]

under the control of different genes [10]. Differentiation-inducing protein MGI-2, but not growth-inducing protein MGI-1, is a DNA-binding protein [79].

These macrophage and granulocyte inducers can be proteins or glycoproteins, depending on the cells in which they are produced, and the presence of carbohydrates does not appear to be necessary for their biologic activity [31]. Their molecular weights are mostly around 23 000 or multiples of this number [48, 55, 64, 65], and MGI-2 activity is more sensitive to proteolytic enzymes and high temperature than MGI-1 activity [31]. MGI-2 has a shorter half-life in serum than MGI-1 [43]. The ready separability of the different forms of MGI seems to depend on the cells from which they are derived [48]. Further studies should determine whether different forms of MGI are derived from a common precursor, and whether tumor cells with the appropriate gene rearrangements, and possibly even normal cells under certain conditions, may produce hybrid molecules of different forms of MGI, including hybrid molecules with MGI-1 and MGI-2 activity [33].

C. Control of Growth and Differentiation in Leukemia

Normal myeloid precursor cells isolated from bone marrow [37] require an external source of MGI-1 for cell viability and growth. There are, however, myeloid leukemic cells that no longer require MGI-1 for viability and growth, so that these leukemic cells can then multiply in the absence of MGI-1 [64, 66]. This gives the leukemic cells a growth advantage over the normal cells when there is a limiting amount of MGI-1. Starting with a decreased requirement for MGI-1, this eventually leads to a complete loss of this requirement. Other myeloid leukemic cells constitutively produce their own MGI-1 [54, 56] and these leukemic cells also have a growth advantage compared with normal cells that require an external source of MGI-1 (Fig. 1). A change in the requirement of MGI-1 for growth, either a partial or complete loss of this requirement, or the constitutive production of MGI-1, thus both give a growth advantage to leukemic cells.

The existence of myeloid leukemic cells that either no longer require MGI-1 for viability and growth or constitutively produce their own MGI-1, raises the question

Type of myeloid cells	Requirement of MGI-1 for growth
Normal	External source
Leukemic	Decrease ⟶ no requirement or Constitutive production

Fig. 1. Differences in MGI-1 requirement for growth in normal and leukemic myeloid cells

whether these leukemic cells can still be induced to differentiate to mature cells by the normal differentiation-inducing protein MGI-2. This question has been answered by showing that there are clones of myeloid leukemic cells that no longer require MGI-1 for growth, but can still be induced to differentiate normally to mature macrophages and granulocytes by MGI-2 via the normal sequence of gene expression; see [64–68]. These mature cells are then no longer malignant in vivo [11, 43, 47]. Injection of these myeloid leukemic cells into embryos has shown that after such injection the leukemic cells can participate in hematopoietic differentiation in apparently healthy adult animals [18, 78].

Injection of MGI-2 into animals, or in vivo induction of MGI-2 by a compound that induces the production of this differentiation-inducing protein, results in an inhibition of leukemia development in animals with such leukemic cells [43, 47]. There are also myeloid leukemic cells that constitutively produce their own MGI-1 and that can be induced to differentiate by MGI-2. Our results indicate that induction of normal differentiation in myeloid leukemic cells by MGI-2 can be an approach to therapy based on the induction of normal differentiation in malignant cells [14, 40, 43, 46, 47, 57]. There are various forms of MGI-2 which differ in their ability to induce differentiation in different clones of myeloid leukemic cells [40, 43, 46, 47].

Leukemic clones that can be induced to differentiate to mature cells by MGI-2 have been found in different strains of mice [5, 14, 15, 25, 28, 38]. They are referred to as MGI^+D^+ (MGI^+ to indicate that they can be induced to differentiate by MGI-2; D^+ for differentiation to mature cells). MGI^+D^+ leukemic cells have specific chromosome changes compared with normal cells [2, 19]. These chromosome changes thus seem to involve changes in genes other than those involved in the induction of normal differentiation. There are other clones of myeloid leukemic cells that can also grow without adding MGI-1, but that are either partly (MGI^+D^-) or almost completely (MGI^-D^-) blocked in their ability to be induced to differentiate by MGI-2 [15, 21, 23, 28, 48, 69, 70]. These differentiation-defective clones have specific chromosome changes compared with MGI^+D^+ cells [2, 19].

There are a variety of compounds, other than MGI-2, that can induce differentiation in MGI^+D^+ clones. Not all these compounds are active on the same MGI^+D^+ clone, and they do not all induce the same differentiation-associated properties. The inducers include certain steroids, lectins, polycyclic hydrocarbons, tumor promoters, lipopolysaccharides, X-irradiation, and compounds used in cancer chemotherapy [42, 64].

The existence of clonal differences in the ability of X-irradiation and cancer

chemotherapeutic chemicals to induce differentiation may help to explain differences in response to therapy in different individuals [64]. As a result of these experiments, we have suggested that it may be possible to introduce a form of therapy based on induction of differentiation [14, 40, 42, 43, 57, 63–65]. This would include prescreening in culture to select for the most effective compounds, and using these compounds for a low dose chemotherapy protocol aimed at inducing cell differentiation [42]. Since different myeloid leukemic clones respond differently to MGI-2 and other compounds, such differences will also occur in leukemic cells from different patients. Based on these suggestions [63, 64], some encouraging clinical results have been obtained with the use of low dose cytosine arabinoside [3, 24, 52].

D. Alternative Pathways of Differentiation

Some of the compounds that induce differentiation in susceptible clones of MGI^-D^+ leukemic cells, including lipopolysaccharide, phorbol ester tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and nitrosoguanide, can induce the production of MGI-2 in these clones. These compounds thus induce differentiation by inducing in the leukemic cells the endogenous production of the normal differentiation-inducing protein MGI-2 [10, 41, 80]. Other compounds such as the steroid dexamethasone, can induce differentiation in MGI^+D^+ clones without inducing MGI-2 [10]. This steroid induces differentiation by other pathways of gene expression than MGI-2 [7, 38]. The same applies to dimethylsulfoxide (DMSO).

Induction of differentiation in some myeloid leukemic clones requires combined treatment with different compounds [30, 39, 41, 74, 75]. In these cases, one compound induces changes not induced by the other, so that the combined treatment results in new gene expression. This complementation of gene expression can occur both at the level of mRNA production and mRNA translation [22]. With the appropriate combination of compounds, we have been able to induce all our MGI^-D^- leukemic clones for some differentiation-associated properties [74, 75]. It will be interesting to determine whether the same applies to differentiation of erythroleukemic cells [16, 50]. It is possible that all myeloid leukemic cells no longer susceptible to the normal differentiation-inducing protein MGI-2 by itself, can be induced to differentiate by choosing the appropriate combination of compounds to give the required complementation. This can include the use of hormones such as steroids [35, 36], or insulin [73, 74], and different nonphysiologic compounds [64], with or without MGI-2.

E. Coupling of Growth and Differentiation in Normal Cells

We have developed a simple procedure for isolating normal myeloid precursor cells from the bone marrow [37]. Incubation of isolated normal myeloid precursors with MGI-1, either MGI-1M or MGI-1G [48], induces the viability and growth of these normal precursors, and results in cell differentiation to macrophages or granulocytes, even without adding the differentiation-inducing protein MGI-2. The incubation of normal myeloid precursors with MGI-1 also results in the induction of MGI-2 [33, 44, 45, 66]. This induction of MGI-2 can be detected as early as 6 h after the addition of MGI-1 [44]. This induction of MGI-2 by MGI-1 can thus account for the induction of differentiation after adding MGI-1 to the normal cells. The induction of differentiation-inducing protein MGI-2 by growth-inducing protein MGI-1 thus appears to be an effective control mechanism for coupling growth and differentiation in the normal cells.

It has been shown that the receptor for epidermal growth factor has tyrosine-specific protein kinase activity [76]. This has also been found for receptors for other growth factors such as insulin [29] and presumably also applies to the receptor for the myeloid cell growth-inducing protein MGI-1. The myeloid differentiation-inducing protein MGI-2, but not MGI-1, can bind to cellular DNA [79]. This shows that growth

and differentiation in normal myeloid cells are coupled by induction of a differentiation-inducing, DNA-binding protein by a growth-inducing protein. This mechanism for coupling growth and differentiation may also apply to other types of cells. Differences in the time of the switch-on of the differentiation inducer would produce differences in the amount of multiplication before differentiation. The platelet-derived growth factor is structurally related to the simian sarcoma virus oncogene *sis* [9, 77]. It will be interesting to determine whether MGI-1 and MGI-2 are structurally related to any of the known oncogenes.

The multiplication of normal cells is regulated at two control points. The first control is that which requires MGI-1 to produce more cells that can then differentiate by the MGI-2 induced by MGI-1. The second control is the stopping of cell multiplication that occurs as part of the program of terminal differentiation to mature cells induced by MGI-2. There is thus a coupling of growth and differentiation in normal cells at both these points.

F. Uncoupling of Growth and Differentiation in Leukemia

As pointed out already, there are MGI^+D^+ clones of myeloid leukemic cells that no longer require MGI-1 for growth, but can still be induced to differentiate normally by MGI-2. These leukemic cells have thus uncoupled the normal requirement for growth from the normal requirement for differentiation. Experiments on the properties of these cells after induction of differentiation by MGI-2 have shown that the normal requirement for MGI-1 for cell viability and growth is restored in the differentiating leukemic cells [13, 44, 45]. MGI-1 added to normal myeloid precursors induces the production of MGI-2, so that the cells can then differentiate by the endogenously produced MGI-2. However, in these leukemic cells, MGI-1 did not induce the production of MGI-2 even though, like normal cells, they again required MGI-1 for viability and growth. There was therefore no induction of differentiation after adding MGI-1 [44, 45]. There is another type of leukemic cell that constitutively produces its own MGI-1 and can also show this lack of induction of MGI-2 by MGI-1, so that the cells do not differentiate [72]. The absence of induction of MGI-2 by MGI-1 therefore uncouples growth and differentiation in these leukemic cells. The lack of requirement of MGI-1 for growth and the absence of induction of the differentiation-inducing protein MGI-2 by the growth-inducing protein MGI-1, are thus mechanisms that uncouple growth and differentiation in MGI^+D^+ leukemic cells [44, 45, 66, 72].

In leukemic cells with constitutive production of MGI-1, changes in specific components of the culture medium can result in an autoinduction of differentiation owing to the restoration of the induction of MGI-2 by MGI-1, which then restores the normal coupling of growth and differentiation (Fig. 2). These changes in the culture medium include the use of mouse or rat serum instead of horse or calf serum, serum-free medium, and removal of transferrin from serum-free medium [72]. Autoinduction of differentiation in this type of leukemic cell may also occur under certain conditions in vivo.

This coupling of growth and differentiation in normal cells is regulated at two control points. The uncoupling of growth and differentiation in MGI^+D^+ leukemic cells is at the first control point, but the coupling at the second control in normal cells, between the induction of differentiation by MGI-2 and the stopping of multiplication in the mature cells, is maintained. There are differentiation-defective MGI^+D^- leukemic cells, that, like the MGI^+D^+ leukemic cells, no longer require addition of MGI-1 for growth. However, in these cells MGI-2 induces only a partial differentiation, mature cells are not produced, and the cells do not stop multiplying. In addition to uncoupling growth and differentiation at the first control point, MGI^+D^- leukemic cells thus show a second uncoupling between the initiation of differentiation by MGI-2 and the stopping of cell multiplication that occurs as part of the normal program of terminal differentiation. It has been suggested that leukemia originates by uncoupling the first control and

Type of myeloid cells	Requirement of MGI-1 for growth	Induction of MGI-2 by MGI-1	Differentiation
Normal	+	Production of MGI-2 →	+
Leukemic	+ or −	No production of MGI-2	−
	Constitutive production MGI-1	Production of MGI-2 →	+ *

*Autoinduction of differentiation under specific conditions

Fig. 2. Differences in induction of differentiation-inducing protein MGI-2 by growth-inducing protein MGI-1 in normal and leukemic myeloid cells

that uncoupling of the second control then results in a further evolution of leukemia [64, 66].

G. Constitutive Gene Expression in Malignancy

Since there are leukemic cells which, unlike normal myeloblasts, no longer require MGI-1 for cell viability and growth, the molecular changes required for viability and growth that have to be induced in the normal cells are constitutive in these leukemic cells. This also applies to leukemic cells that constitutively produce their own MGI-1. This suggests that the origin of myeloid leukemia can be due to a change from an induced to a constitutive expression of genes that control cell viability and growth [64, 66].

Studies on changes in the synthesis of specific proteins in normal myeloblasts, MGI^+D^+, MGI^+D^-, and MGI^-D^- leukemic clones at different times after adding MGI-1 and MGI-2, using two-dimensional gel electrophoresis [32], have directly shown that there have been changes from inducible to constitutive gene expression in the leukemic cells. The results also indicate a relationship between constitutive gene expression and uncoupling of the induction of differentiation by MGI-2 and the stopping of multiplication in the mature cells. The results indicate that changes from an induced to a constitutive expression of certain genes are associated with the uncoupling of growth and differentiation, both at the control which requires MGI-1 to produce more cells and at the control of the stopping of cell multiplication that occurs in the formation of mature cells.

The protein changes during the growth and differentiation of normal myeloblasts seem to be induced by MGI-1 and MGI-2 as a series of parallel multiple pathways of gene expression [32]. It can be assumed that the normal developmental program that couples growth and differentiation in normal cells requires synchronous initiation and progression of these multiple parallel pathways. The presence of constitutive gene expression for some pathways can be expected to produce asynchrony in the coordination required for the normal development program. Depending on the pathways involved, this asynchrony could then result in an uncoupling of the controls for growth and differentiation and produce different blocks in the ability to be induced for the differentiation process and to terminate it.

We have been able to treat MGI^-D^- leukemic cells so as to induce the reversion of specific proteins from the constitutive to the nonconstitutive state. This reversion was then associated with a gain of inducibility by MGI-2 for various differentiation-associated properties. Reversion from the

constitutive to the nonconstitutive state in these cells thus restored the synchrony required for induction of differentiation [75].

The suggestion derived from these results [32, 64, 66] is, therefore, that myeloid leukemia originates by a change that produces certain constitutive pathways of gene expression, so that cells no longer require MGI-1 for growth or constitutively produce MGI-1 without inducing MGI-2. These leukemic cells can, however, still be induced to differentiate normally by MGI-2 added exogenously or induced in the cells in other ways. The differentiation program induced by MGI-2 can thus proceed normally when it is uncoupled from the growth program induced by MGI-1. This can be followed by constitutive expression of other pathways, resulting in the uncoupling of other controls and an asynchrony that interferes with the normal program of terminal differentiation. These second changes then result in the further evolution of leukemia [66].

H. Reversal of Malignancy by Induction of Differentiation in Various Types of Tumors

These conclusions on the origin and evolution of myeloid leukemia may be applicable to malignant tumors derived from other types of cells whose viability, growth, and differentiation are induced by other physiologic inducers. Identification of the physiologic inducers of growth and differentiation for different cell types would be a crucial requirement in extending these conclusions to those other tumors. However, even in the absence of such identifications, it appears likely that teratocarcinoma cells [8, 53] may be comparable to MGI^+D^+ myeloid leukemic cells. The presence of fetal proteins in certain tumors may also be due to constitutive gene expression in the tumor of a protein that is induced by the physiologic inducer during the developmental program in the normal fetus [66]. There are probably a variety of tumors in which: (a) the original malignancy has a normal differentiation program and the cells are malignant because of uncoupling of the requirement for growth from the requirement for differentiation by changing the gene expression required for growth from inducible to constitutive; and (b) where the further evolution of the tumor results from changes from inducible to constitutive of other pathways of gene expression that produce asynchrony in the normal differentiation program, so that mature nondividing cells are not formed by the physiologic inducer of differentiation. However, even these tumors may still be induced to differentiate to form nonmalignant cells by treatment with compounds that can reverse the constitutive to the nonconstitutive state or induce the differentiation program by other pathways. In some tumors, such as sarcomas, reversal of malignancy can be obtained by specific changes in the karyotype [20, 61–63, 81]. But the stopping of cell division in mature cells by inducing differentiation induces a reversion of malignancy by bypassing the genetic changes that produce the malignant phenotype.

Acknowledgments. This research is now being supported by a contract with the National Foundation for Cancer Research, Bethesda, and by grants from the Jerome A. and Estelle R. Newman Assistance Fund, and the Julian Wallerstein Foundation.

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