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Human granulocyte–macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin

(cell proliferation)

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ABSTRACT **Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a member of a family of glycoprotein hormones that stimulate the proliferation and differentiation of hemopoietic cells *in vitro* and *in vivo*. We now report that human GM-CSF can also stimulate the proliferation of two osteogenic sarcoma cell lines, a breast carcinoma cell line, a simian virus 40-transformed marrow stromal cell line, and normal marrow fibroblast precursors. These findings suggest a more general regulatory function of GM-CSF on nonhemopoietic cell types than previously anticipated. They also raise the possibility of adverse side effects of GM-CSF therapy in patients whose malignant cells may be directly stimulated by this molecule and suggest a previously unanticipated role of GM-CSF gene activation in the evolution of solid tumors and in the pathogenesis of myelofibrosis.**

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a glycoprotein first identified by its presence in crude preparations of conditioned medium that stimulate immature hemopoietic cells to proliferate and differentiate *in vitro* into mature granulocytes and macrophages (reviewed in ref. 1). Both murine and human GM-CSF have been purified to homogeneity (2, 3) and their genes cloned and expressed (4–6). The resultant availability of large amounts of purified recombinant protein has allowed a wider range of primitive (7, 8) and fully differentiated (3, 9) cell types within the hemopoietic system to be recognized as directly GM-CSF responsive than was initially appreciated. The ability of GM-CSF to markedly stimulate the production (and function) of granulocytes and macrophages *in vivo* (10, 11), suggested that GM-CSF administration might be useful clinically in situations in which the number and/or functional capacity of leukocytes is suboptimal. Initial results in cancer patients undergoing intensive therapy (12), acquired immunodeficiency syndrome (AIDS) patients (13), and patients with myelodysplasia (14) have been very encouraging. However, as part of the evaluation of such applications of GM-CSF therapy, it would be important to identify any other effects that GM-CSF might have on various types of normal or malignant cells of nonhemopoietic origin.

It has recently been shown that the process of bone resorption and remodeling is substantially affected by cytokines released by hemopoietic cells. Since these responses involve both osteoclasts and osteoblasts, their separate responses to growth factors are of interest. Interleukin 1 (15) and tumor necrosis factor (16) both stimulate bone resorption. The effect of hemopoietic growth factors on proliferation and differentiation of osteoblasts has, however, not been investigated, although interleukin 1 has been shown to inhibit the proliferation of mouse osteoblast-like cells (17). Since osteoblasts respond to mitogens present in serum, we used a defined serum-free but supportive medium to examine the effect of various cytokines on a human osteogenic sarcoma cell line (MG-63) (18). We report here that the proliferation of these human osteogenic sarcoma cells can be stimulated by highly purified recombinant human GM-CSF, and that this response can also be demonstrated in other human cells of nonhemopoietic origin.

MATERIALS AND METHODS

Materials. Recombinant human GM-CSF expressed in *Escherichia coli* was obtained from Biogen (Cambridge, MA) and from Behringwerke (Marburg, F.R.G.). The material from Biogen was 93% pure and contained 10^{-6} ng of endotoxin per ng of GM-CSF by the limulus assay gel test. It was supplied to us containing 1 mg of human serum albumin per 100 μg of GM-CSF. The material from Behringwerke was clinical grade material, >99% pure, and did not contain any additives or detectable endotoxin. Glycosylated recombinant human GM-CSF expressed in yeast, purified to homogeneity, and supplemented with 1 mg of bovine serum albumin per 100 ng of GM-CSF was purchased from Genzyme (Boston). All GM-CSF preparations were diluted in Iscove's medium (Sigma) containing 1% (vol/vol) deionized bovine serum albumin (Sigma). Purified neutralizing anti-human GM-CSF monoclonal antibody was purchased from Genzyme and diluted in the same diluent. [^{3}H]Thymidine (2 μCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham. Insulin/transferrin/selenium (ITS) was obtained from Collaborative Research (Bedford, MA). MG-63, HOS, SK-N-SH, SK-N-MC, and MCF-7 cells were obtained from the American Type Culture Collection. CFU-ST clone 16 cells are simian virus 40-immortalized human marrow "fibroblasts" isolated, cloned, and maintained in this laboratory (19).

Cell Line Maintenance. MG-63 osteosarcoma and their variants, MG-63 3A cells (20), HOS osteosarcoma (21), and SK-N-SH and SK-N-MC neuroblastoma cells were passaged in Dulbecco's minimal essential medium (DMEM) (GIBCO) containing 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml). MCF-7 breast carcinoma cells (22) were passaged in RPMI 1640 medium (GIBCO) in 10% fetal bovine serum. CFU-ST clone 16 cells were maintained in α-medium (Sigma) plus 10% fetal bovine serum. For routine subculturing, cell monolayers were washed with phosphate-buffered saline (150 mM Na-

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Abbreviation: GM-CSF, granulocyte–macrophage colony-stimulating factor.

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Cl/10 mM sodium phosphate, pH 7.3), and detached with 1 mM EDTA or trypsin.

Cell Line Proliferation Assays. Cells from monolayer cultures were washed three times in serum-free DMEM and resuspended at a concentration of 10^5 cells per ml in serum-free DMEM/Ham's F-12 medium (1:1) containing ITS (Collaborative Research). For [^{3}H]thymidine incorporation measurements, the cells were then plated into flat-bottomed tissue culture 96-well microtiter plates at 10^4 cells per well. After 4 hr, the above medium, with or without the additions indicated, was added together with sufficient [^{3}H]thymidine (2 Ci/mmol) to give a final concentration of 1 μCi per 200 μl. [^{3}H]Thymidine incorporation was measured after 20 hr at 37°C. The cells were detached with 10 mM EDTA and harvested onto glass-fiber cellulose discs with a cell harvester (Skatron, Lierbyen, Norway). The discs were allowed to dry and the radioactivity was determined by liquid scintillation counting. ^{3}H dpm shown are those obtained after subtracting the dpm obtained in serum-free medium controls. For cell number measurements, cells were plated into 24-well flat-bottomed tissue culture plates at a cell density of 10^5 cells per well. Cell numbers were determined by detaching the cells with EDTA (5 mM) and counting on a Coulter Counter.

Human Marrow Cultures. Normal marrow aspirates were obtained with informed consent from individuals undergoing marrow harvesting for either autologous or allogeneic bone marrow transplantation. Fibroblast precursor colony-forming cell assays were performed essentially as described (23) by plating light-density (<1.077 g/ml) marrow cells into tissue culture dishes containing α-medium plus 10% fetal bovine serum (2.6×10^4 cells per cm^2 surface area). Dishes were incubated undisturbed for 1–2 weeks and then adherent fibroblast colonies were counted after fixation and staining with May–Grünwald–Giemsa stain. For assessment of GM-CSF activity on human granulocyte–macrophage colony-forming cells, nonadherent light-density (<1.077 g/ml) cells from 1-week-old long-term human marrow cultures (24) were assayed in standard methylcellulose cultures (24) at a final concentration of 5×10^4 cells per ml. Granulopoietic colonies containing >20 cells were scored after 3 weeks.

RESULTS

Highly purified recombinant human GM-CSF significantly increased [^{3}H]thymidine incorporation into MG-63 cells (Fig. 1). To determine whether the stimulation of [^{3}H]thymidine incorporation into MG-63 cells was related to an increase in cell number, we also performed cell counts. As shown in Fig. 2, GM-CSF shortened the population doubling time of MG-63 cells over a period of at least 7 days, so that after 1 week there were approximately twice as many MG-63 cells in GM-CSF supplemented cultures by comparison to control cultures. Two other preparations of highly purified recombinant human GM-CSF were found to be similarly active on MG-63 cells (Table 1). The specificity of the stimulatory effect of both GM-CSF preparations was confirmed by using a neutralizing anti-human GM-CSF monoclonal antibody. As shown in Table 1, addition of sufficient antibody completely neutralized the ability of highly purified recombinant GM-CSF to stimulate MG-63 cells (Exp. 1, group e). However, this neutralizing effect was reversed by the addition of excess antigen—i.e., excess GM-CSF (group f), indicating that the action of the antibody preparation used in these experiments was due to its reactivity with human GM-CSF. Since small amounts of endotoxin were detected in one of the GM-CSF preparations, we also tested whether the presence of endotoxin might mimic the GM-CSF effect observed on MG-63 cells. As shown in Table 1, endotoxin did not stimulate the proliferation of these cells.

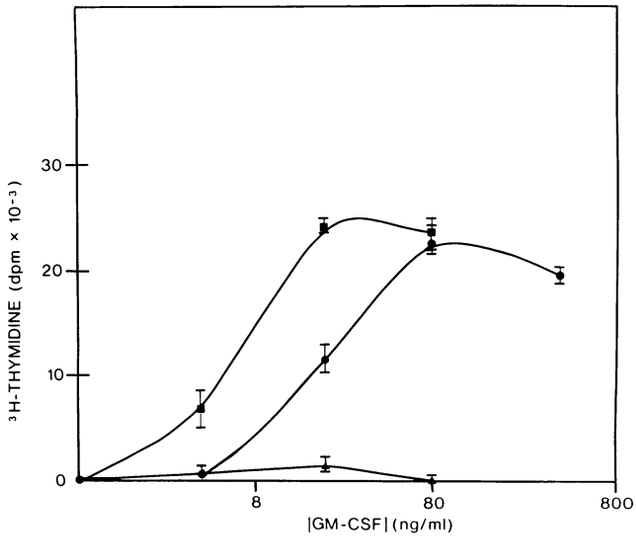

FIG. 1. Dose-dependent stimulation by human recombinant GM-CSF (Biogen) of [^{3}H]thymidine incorporation into MG-63 human osteosarcoma cells (●), MCF-7 human breast carcinoma cells (■), and lack of effect of SK-N-SH human neuroblastoma cells (▲). Each value represents the mean ± 1 SEM of dpm from three replicates after subtracting the dpm measured in the absence of added GM-CSF. Control values (no GM-CSF) were 6480 ± 220 dpm for MG-63 cells, 9615 ± 520 dpm for MCF-7 cells, and 16,420 ± 850 dpm for SK-N-SH cells in the experiments shown.

To determine whether the stimulation of cell proliferation by GM-CSF was a response unique to MG-63 cells, several other human cell types were examined under the same serum-free conditions and [^{3}H]thymidine incorporation end point. These experiments revealed that GM-CSF could also stimulate HOS cells, an osteosarcoma cell line (21), MCF-7 breast carcinoma cells (22), and CFU-ST clone 16 cells, a recently isolated and cloned line of simian virus 40-transformed marrow stromal cells (19), all in a similar dose-dependent manner (Fig. 1 and Table 2). The response of MCF-7 cells was also reproduced with a different GM-CSF preparation (from Genzyme) and the results were confirmed by using viable cell counts as the end point (data not shown).

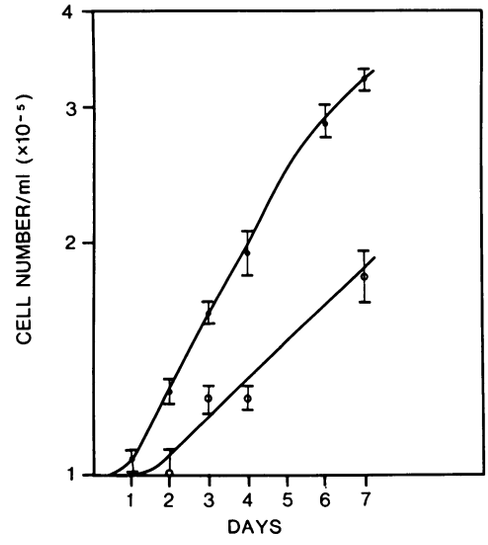

FIG. 2. Effect of GM-CSF on the cell proliferation of MG-63 osteosarcoma cells. GM-CSF at a final concentration of 80 ng/ml was added to some of the wells (●) and not to others (○). Values shown are the means ± 1 SEM of counts obtained from triplicate wells.

Table 1. Effect of human GM-CSF and endotoxin on MG-63 cell proliferation

Group	Addition: Stimulant	Addition: Anti-GM-CSF antibody	[^{3}H]Thymidine incorporation, dpm per 20 hr	% inhibition
		Experiment 1		
a	None		3824 ± 852	
b	None	Anti-GM-CSF (20 μg/ml)	3225 ± 773	
c	Endotoxin* (0.1 ng/ml)		856 ± 120	
d	GM-CSF (10 ng/ml) (Behringwerke)		8024 ± 140	
e	GM-CSF (10 ng/ml) (Behringwerke)	Anti-GM-CSF (20 μg/ml)	2723 ± 277	100
f	GM-CSF (100 ng/ml) (Behringwerke)	Anti-GM-CSF (20 μg/ml)	6152 ± 1122	23
		Experiment 2		
a	None		2045 ± 300	
b	None	Anti-GM-CSF (3.2 μg/ml)	1940 ± 817	
c	GM-CSF (10 ng/ml) (Genzyme)		9727 ± 540	
d	GM-CSF (10 ng/ml) (Genzyme)	Anti-GM-CSF (0.4 μg/ml)	4714 ± 270	64
e	GM-CSF (80 ng/ml) (Biogen)		6618 ± 200	
f	GM-CSF (80 ng/ml) (Biogen)	Anti-GM-CSF (3.2 μg/ml)	4471 ± 151	46

Anti GM-CSF antibody was added directly (control) or preincubated with the GM-CSF to be added for 2 hr at 4°C prior to addition to the cells. In human marrow granulocyte–macrophage colony-forming cell assays, 20 μg of this anti-GM-CSF antibody preparation per ml was just sufficient to completely eliminate the formation of any GM colonies in methylcellulose assays of nonadherent cells containing 1 ng of purified recombinant human GM-CSF per ml and this effect could be completely reversed by 10 ng of GM-CSF per ml.
*Endotoxin standard in the Sigma endotoxin detection kit. The endotoxin content of the serum-free medium used in the assay was 0.04 ng/ml.

There was some heterogeneity in the sensitivity of the responsive cell lines to GM-CSF, and all appeared 10–20 times less sensitive than normal human granulopoietic progenitor cells tested in a colony assay (Fig. 3). Nevertheless, these findings indicate that GM-CSF is not restricted in its action to either hemopoietic or nonhemopoietic cells of mesodermal derivation, but it can stimulate the growth of cells of widely diverse embryonic origins. Interestingly, GM-CSF did not have any effect on the incorporation of [^{3}H]thymidine into MG-63.3A cells (20), an osteoblast-like, but more differentiated, variant of MG-63 cells. GM-CSF also failed to stimulate two neuroblastoma cell lines (SK-N-SH and SK-N-MC; Table 2). Thus, GM-CSF is not a general growth factor for all transformed cells. GM-CSF was also able to enhance significantly the number (by a factor of 2.4 ± 0.3; three experiments) and size of colonies derived from "fibroblast" precursors present in fresh human marrow even in the presence of 10% serum (Fig. 4). While this latter effect could be due to an indirect mechanism (since many GM-CSF responsive hemopoietic cells are also present in these primary cultures), the fact that an immortalized derivative of these cells (CFU-ST clone 16) is also GM-CSF responsive suggests that the normal parent cell also has this capacity.

Additional studies showed that the ability of GM-CSF to stimulate [^{3}H]thymidine incorporation into MG-63 cells was independent of the presence of added albumin (0.4 mg/ml) (data not shown) or the ITS serum substitute (Table 2). The latter result suggests that the response of MG-63 cells to GM-CSF does not depend on their cycling status. Indeed, it appears that GM-CSF alone can replace the requirement for ITS at least over the short term. In contrast, the level of [^{3}H]thymidine incorporation into MG-63 cells maintained in the same medium with 10% fetal calf serum (but no ITS) was unchanged when GM-CSF was also added (Table 2). It seems likely that the marked stimulatory effect of fetal calf serum on MG-63 cells may mask or supersede their response to GM-CSF, since GM-CSF, although mitogenic for these cells, appears to be much weaker in this respect than fetal calf serum.

DISCUSSION

Our findings raise a number of interesting possibilities concerning the biological role of GM-CSF and the significance of GM-CSF gene regulation. Previous studies have suggested that GM-CSF can be produced by a variety of normal cells including macrophages, T lymphocytes, fibroblasts, and endothelial cells but generally only after their activation (reviewed in ref. 25). The data presented here suggest that responsiveness to GM-CSF is also a function of many cell types. This property can thus clearly no longer be viewed as a marker of cells derived from a hemopoietic precursor (26, 27). GM-CSF may in fact have a broad role as a normal regulator of nonhemopoietic as well as hemopoietic cells. For example, since osteoclasts and macrophages are believed to be related (28), GM-CSF may serve as a molecular link in the mechanisms that coordinate bone resorption by activated osteoclasts and the production of new bone by osteoblasts.

GM-CSF may also be involved in the stimulation of nonhemopoietic malignant cell growth. Infiltrates of acti-

Table 2. Effect of GM-CSF on proliferation of a variety of human cell lines

Cell line tested	^{3}H dpm: Control	^{3}H dpm: + rGM-CSF (80 ng/ml)	-fold change
MG-63	8,595 ± 1100	32,885 ± 1,800	3.8
MG-63 − ITS	2,346 ± 200	28,103 ± 420	11.8
MG-63 + 10% fetal calf serum	137,000 ± 4300	132,870 ± 13,300	None
MCF-7	16,494 ± 140	41,021 ± 1,400	2.5
CFUST clone 16	25,622 ± 40	34,713 ± 1,550	1.4
HOS	64,575 ± 6100	100,838 ± 5,500	1.6
SK-N-SH	65,110 ± 2100	65,397 ± 1,030	None
SK-N-MC	26,095 ± 1150	24,833 ± 1,700	None
MG-63.3A	2,743 ± 670	2,687 ± 390	None

r, Recombinant.

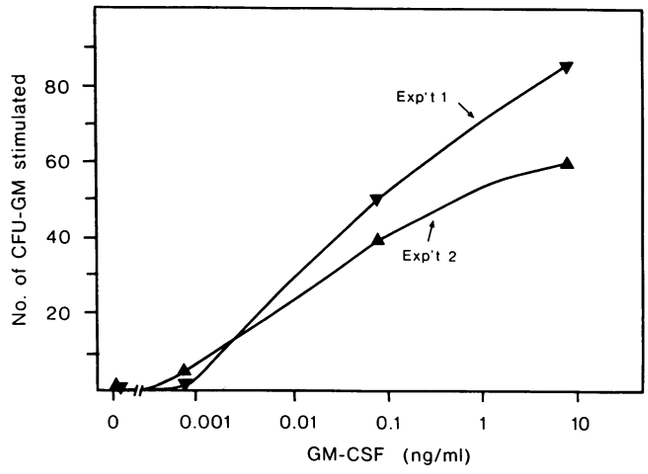

FIG. 3. Titration of the ability of human GM-CSF (Biogen) to stimulate granulocyte–macrophage colony-forming cells (CFU-GM) in assays of 5×10^4 nonadherent human marrow cells cultured in standard methylcellulose cultures. Similar results were obtained for the two other GM-CSF preparations used in this study. Values shown represent the mean colony counts from two replicate assays.

vated macrophages and lymphocytes are well established features of many tumors and the ability of these cells to release undefined factors that can stimulate the proliferation of malignant cells is well known (29–31). The present studies identify GM-CSF as a potential mediator of this activity. Although the cell lines studied here appear less sensitive to GM-CSF than hemopoietic precursors, it is now known that GM-CSF can be specifically bound to components of the extracellular matrix (32, 33). This likely serves as a mechanism for concentrating this growth factor in tissues *in vivo* and may allow very high levels to be achieved in different sites, particularly if locally produced. Involvement of GM-CSF in autocrine mechanisms of growth by nonhemopoietic malignant cells should also now be considered. A number of human tumors of nonhemopoietic origin have been identified that can constitutively release hemopoietic growth factors including GM-CSF (e.g., see ref. 34). This suggests the possibility that the recently described role of GM-CSF as an autocrine stimulator of some human acute leukemia cells (35) may also apply to some nonhemopoietic cancers. The abnormal activation of GM-CSF gene expression might thus constitute a mechanism contributing to malignant progression in a variety of cell types. The ability of osteoclasts as well as marrow stromal cells to respond to GM-CSF might also explain some of the secondary effects observed in cancer patients in which inappropriate production of GM-CSF would be a candidate mechanism of autocrine growth (for example, bone resorption in breast cancer and myelofibrosis in certain myeloproliferative diseases such as chronic myeloid leukemia). To investigate these possibilities, additional experiments will be required to establish whether the *in vitro* responses detected here can also be demonstrated *in vivo*. Such experiments will also be important to determine the potential hazards of using GM-CSF therapy in patients. In this regard, it is interesting that a recent study of transgenic GM-CSF mice indicates a number of systemic effects of continuously elevated GM-CSF production (36), which may not be due exclusively to excess macrophage numbers.

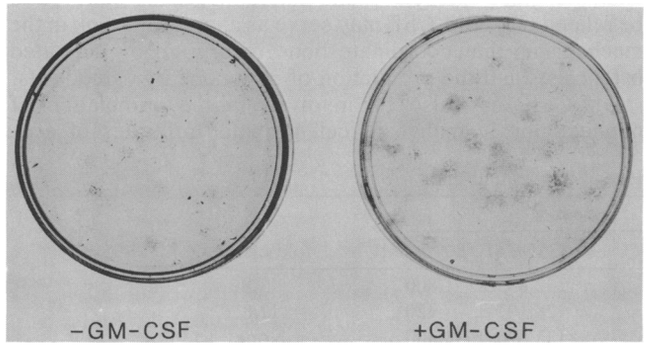

FIG. 4. Effect of GM-CSF (Biogen) on fibroblast colony formation by precursors present in human marrow. The number of fibroblast colonies in the dish containing GM-CSF is double the number obtained in the control plate. Assessment of cell morphology in the stained dishes showed that all colonies in both types of plates were indistinguishable and resembled typical fibroblast-like cells derived from CFU-F.

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