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GRANULOCYTE COLONY-STIMULATING FACTOR INDUCES NEUTROPHILS TO SECRETE MACROPHAGE COLONY-STIMULATING FACTOR

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In this work we provide evidence showing that granulocytes produce macrophage colony-stimulating factor (M-CSF) from the band cell stage and secrete this factor when induced to differentiate into polymorphonuclear cells by recombinant human granulocyte colony-stimulating factor (rhG-CSF). Using an enriched population of myeloid band cells from murine bone marrow, we identified the presence of M-CSF with a chromophore-labelled monoclonal anti-M-CSF antibody. Using ELISA we detected the secretion of M-CSF in the supernatants of cultures of enriched band cells when induced with rhG-CSF to differentiate into mature neutrophils. We also found that M-CSF is the only factor responsible for the colony forming activity in the supernatants and lysates of band cells treated with rhG-CSF.

The granulocyte colony-stimulating factor (G-CSF) is a glycoprotein secreted by bone marrow stromal cells, macrophages, fibroblasts, and endothelial cells.[1] G-CSF stimulates the survival, proliferation, and differentiation of myeloid progenitor cells towards neutrophilic granulocytes.[2,3] G-CSF deficient mice show chronic neutropenia and a reduced granulopoietic response to infection, indicating that this factor plays an essential role in the regulation of granulopoiesis.[4] G-CSF has been used to stimulate neutrophil recovery in neutropenic mice and in cancer patients after high-dose chemotherapy.[5–8] In these treatments, G-CSF enhanced not only the recovery of neutrophils but also of monocytes,[5,7,9] probably due to the secretion of macrophage colony-stimulating factor (M-CSF).[7] It is known that haematopoietic cells induced to proliferate and differentiate by a growth factor in turn produce and secrete other growth factors,[10,11] for example, when monocyte precursors produce G-CSF when induced to differentiate by granulocyte macrophage-CSF (GM-CSF).[12] On the other hand, the fact that only granulocytes and their precursors express receptors for G-CSF,[13] and that G-CSF is known to induce the formation not only of granulocytes but also of macrophage colonies in vitro,[12,14] suggest the possible secretion of M-CSF by granulocytes treated with G-CSF.

In order to test this last hypothesis, in this study bone marrow band cells were induced to differentiate in the presence of recombinant human G-CSF (rhG-CSF) and the possible secretion of M-CSF by these cells was evaluated.

RESULTS

rhG-CSF differentiates band cells into polymorphonuclear neutrophils

In order to determine if rhG-CSF is able to stimulate differentiation of neutrophils from band cells to polymorphonuclear cells, we cultured band cells-enriched murine bone marrow in the presence of 20 ng/ml of rhG-CSF and the percentage of induced polymorphonuclear cells was evaluated after 24 and 48 h. Our results showed that after 24 h there was a strong induction by rhG-CSF towards polymorphonuclear differentiation (33% from the initial band cell population) (Fig. 1). In the cultures without inducer,

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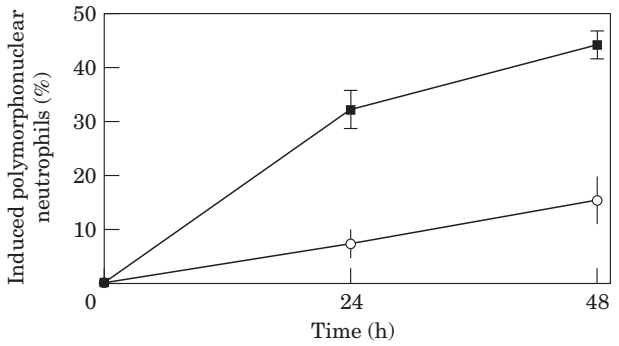

Figure 1. Kinetics of differentiation of band cells from murine bone marrow, towards polymorphonuclear neutrophils from 0 to 48 h of culture (RPMI+10% FBS V/V) with 20 ng/ml rhG-CSF (—■—) or without (—○—) inducer.

The cells were collected at the indicated times, fixed with methanol and stained with Giemsa.

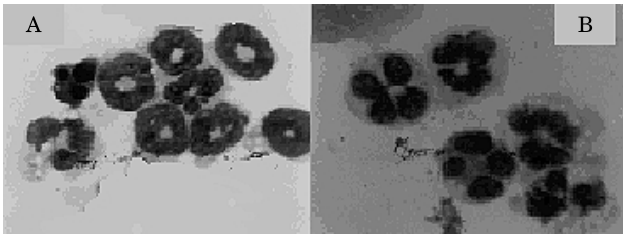

Figure 2. Determination of cell morphology.

Band BMCs cultured with or without 20 ng/ml of rhG-CSF after 48 h of culture with RPMI 1640 medium and 10% FBS (V/V). A, untreated cells; B, treated with rhG-CSF. Magnification: × 400.

differentiation was seen in only 6% of the cells. The incidence of differentiation increases as a function of time in culture, reaching 49% after 48 h of culture in the presence of rhG-CSF (Figs 1 and 2).

Neutrophils treated with rhG-CSF secrete a colony-stimulating factor

In order to determine if the differentiation of band cells into polymorphonuclear cells is associated with the secretion of other colony growth factors, the colony forming capacity of the supernatants and cell lysates of these cells was evaluated in vitro. For this purpose 10^5 bone marrow cells were cultured in the presence of a 20% solution of the supernatants and lysates of band cells precultured for 24 and 48 h in the presence of rhG-CSF. Our results showed that there was an induction of colony formation in the supernatant and lysate from the 48 h band cell cultures treated with rhG-CSF, and that there was a smaller one from band cells that were not induced by this factor (Fig. 3).

As the identity of the factor with the colony forming activity was so far unknown, we evaluated the

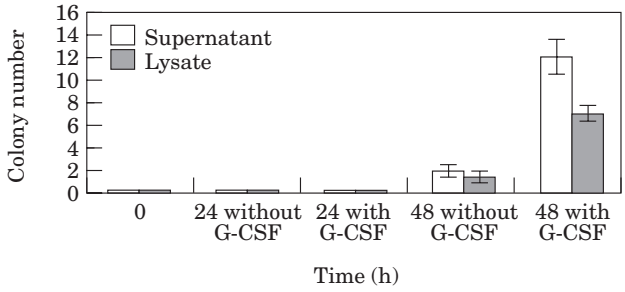

Figure 3. Colony forming assay.

Lysates or supernatants of band neutrophils (10^6 cells per ml) treated or not with 20 ng/ml rhG-CSF during 48 h of culture, were collected and subsequently added (20% V/V) to 10^5 murine bone marrow cells/well in 24-well plates in a colony assay. The number of colonies was scored 7 days after plating.

TABLE 1. Cell morphology of colonies receiving either supernatants or lysates from band cells treated with rhG-CSF

Inducer	Colony type (%)	
	Granulocytes	Macrophages
Without inducer	0 ± 0	0 ± 0
Supernatants	5 ± 2.5	95 ± 2.5
Lysate	0 ± 0	100 ± 0
rhG-CSF	92 ± 2	8 ± 2
rhM-CSF	0 ± 0	100 ± 0

Morphology of cells from murine bone marrow colonies (10^5 cells/well) after 7 days of incubation in the double layer colony assay, stimulated with either supernatants or lysates (20% V/V) from band cells, and cultured for 48 h with or without rhG-CSF (20 ng/ml). The colonies were collected using the technique of transfer onto coverslips, and subsequently stained with Giemsa. The morphology was assessed using an optical microscope. 20 ng/ml rmM-CSF (recombinant murine macrophage-colony stimulating factor) and 20 ng/ml rhG-CSF were used as positive controls. rhG-CSF, recombinant human granulocyte colony-stimulating factor; rhM-CSF, recombinant human macrophage-colony stimulating factor.

morphology of the cells forming the colonies. In assays using supernatants from band cells treated 48 h with rhG-CSF, 95% of the cells in the colonies had a macrophage morphology and 5% corresponded to neutrophils (Table 1). Bone marrow cells receiving lysates of band cells, with the same treatment as above, formed only colonies containing cells with macrophage morphology. rhG-CSF and recombinant murine M-CSF (rmM-CSF) were used as controls. rhG-CSF promoted formation of granulocytes; 92% of the colonies were characterized as belonging to this lineage and 8% were of macrophage morphology. When rmM-CSF was added to the cultures, 100% of the colonies were of the macrophage lineage.

Band cells and polymorphonuclear cells produce M-CSF

The above results strongly suggested that the factor produced by granulocytes treated with rhG-

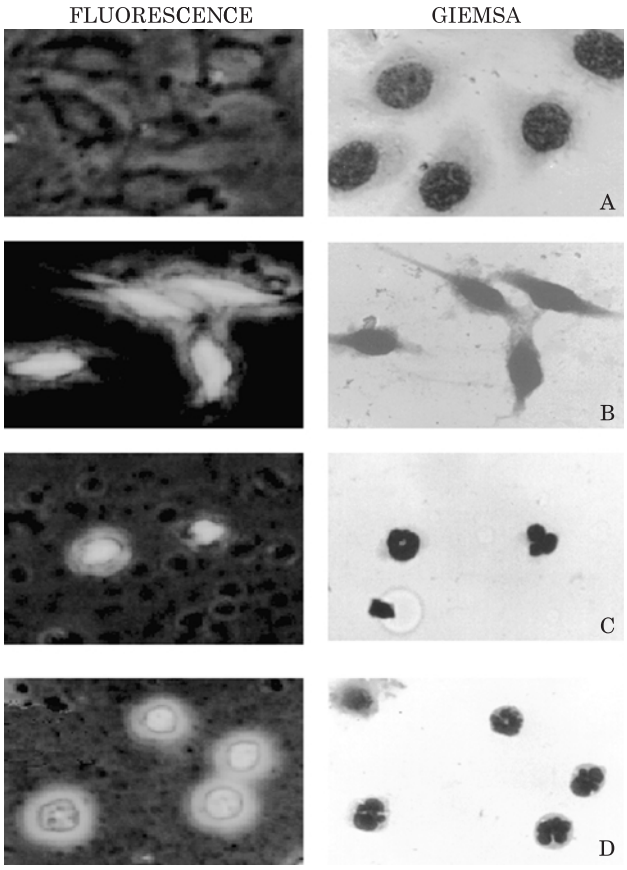

Figure 4. Band cells cultured with and without 20 ng/ml rhG-CSF during 48 h were tested for their capacity to produce M-CSF.

A chromophore-labelled (Atf-Bct-NHS label reagent) monoclonal anti-murine M-CSF antibody was used. After 48 h of culture, 3×10^6 band cells stimulated or not with rhG-CSF were washed twice with PBS, resuspended in RPMI 1640 medium and 10 µl of chromophore-labelled antibodies were added. Once the labelling procedure was performed, cytospin slides were prepared and the cells fixed with methanol. On the left, UV light microscopy was employed, and on the right the same cells stained with Giemsa (10% V/V) to reveal their morphology. (A) A cervix carcinoma cell line (CALO, negative control); (B) L-929 fibroblasts (positive control for M-CSF); (C) Band cells cultured without G-CSF; (D) Band cells cultured in the presence of 20 ng/ml rhG-CSF.

CSF, and responsible for the formation of colonies with macrophage morphology, was M-CSF. We used a chromophore-labelled anti-M-CSF antibody, sensitive to UV light, to determine the presence of M-CSF through microscopy. A cervical carcinoma cell line Calo, which is negative for the presence of M-CSF (Fig. 4A), as well as L-929 cells that are known to be M-CSF producers (Fig. 4B), were used as negative and positive controls, respectively. All the granulocytic cells, whether treated or not with rhG-CSF, stained with the anti-M-CSF antibody (Fig. 4C and D). Using goat IgG anti-murine M-CSF antibodies in an ELISA, we confirmed the presence of M-CSF in band cell cultures with or without rhG-CSF (Fig. 5).

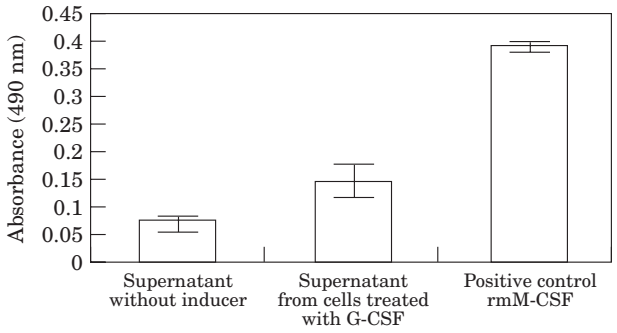

Figure 5. Presence of M-CSF in supernatants of band cells treated with G-CSF for during 48 h was identified by ELISA.

The primary antibody was a goat-anti-mouse IgG anti-M-CSF used at a dilution of 1:200 in BSA/PBS, and the secondary was a peroxidase-labelled rabbit-anti-goat antibody (dilution of 1:3000 in BSA/PBS). Supernatants of band cells without treatment and rmM-CSF were used as negative and positive controls respectively.

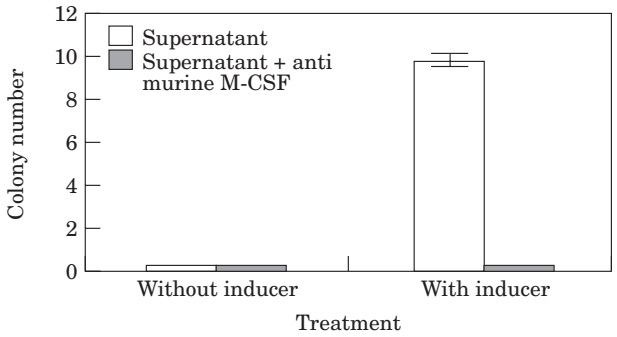

Figure 6. Addition of murine anti-M-CSF antibodies (1:200 dilution in the first agar layer) block the colony stimulating activity on BMCs (10^5 cells/well) of supernatants (20% V/V) from band cells treated during 48 h with G-CSF (20 ng/ml).

No colonies formed in the absence of inducer; when supernatants from band cells were added, colony formation occurred which was inhibited in the presence of anti-M-CSF antibodies.

M-CSF is the only colony forming factor produced by neutrophils cultured in the presence of rhG-CSF

A monoclonal anti-M-CSF was used to assess whether the colony-forming activity was due to the presence of M-CSF identified in supernatants and lysates of cells treated with rhG-CSF. Our results showed that when the cultures received anti-M-CSF colony formation was suppressed (Fig. 6), hinting thus that M-CSF is the only colony stimulating factor produced by neutrophils cultured in the presence of rhG-CSF.

DISCUSSION

In this study we demonstrate that M-CSF production by polymorphonuclear cells is present even at the

band cell stage and that the presence of rhG-CSF is required to promote the secretion of M-CSF. In a previous study we demonstrated that macrophages secrete G-CSF,[12] it is thus interesting that in turn this factor secreted by macrophages induces in granulocytes the secretion of a macrophage growth factor (M-CSF), suggesting a very close interrelationship in the differentiation process of these two cell types.

We believe that the only cells able to produce M-CSF in our cultures after stimulation with rhG-CSF are those of the granulocytic lineage, because several studies demonstrate the absence of receptors for G-CSF in cells other than granulocytes and their precursors.[16,17]

That G-CSF induces M-CSF secretion can explain the fact that when murine bone marrow cells were cultured in agar in the presence of G-CSF there were not only 90% granulocyte colonies, but up to 10% of monocyte–macrophage colonies.[12,14] On the other hand, in neutropenic patients and also in mice treated with 5-fluorouracil (5-FU), there is not only an increase in the number of neutrophils, but there is also recovery in the number of monocytes when G-CSF is administered.[5–7] More recent reports indicate that injection of G-CSF in mice treated with 5-FU promotes the production of M-CSF, a situation that reflects the recovery of precursors of both macrophages (M-CFU) and granulocytes (G-CFU)[7] as supported by our data.

It is already known that some of the first cells to arrive to the site of infection are granulocytes, a type of leukocyte that quickly dies through apoptosis once the task has been achieved,[18] being subsequently eliminated by macrophages.[19] The fact that macrophages show chemotaxis towards M-CSF,[20] and as shown in this work, M-CSF is produced by granulocytes after treatment with rhG-CSF, indicates that stimulation of granulocytes guarantees the presence and accumulation of macrophages in the site of infection through their secretion of M-CSF, and in addition, promotes the elimination of other apoptotic cells.

MATERIAL AND METHODS

Mice

Mice of either sex, strain CD-1, were used at 6–8 weeks of age as donors of bone marrow cells (BMCs).

Cytokines and antibodies

rhG-CSF, rmM-CSF and goat IgG anti-murine M-CSF were purchased from R&D Systems (R&D Systems Inc, MN, USA). Peroxidase-labelled rabbit anti-goat IgG was purchased from DAKO (DAKO, MA, USA).

Cell culture

All BMCs cultures were maintained under a 5% CO_2 atmosphere, at 37°C, and 95% relative humidity. The cells were cultured in RPMI 1640 medium (Gibco Life Technologies, MD, USA), supplemented with 10% (V/V) fetal bovine serum (FBS) (Gibco, USA) previously inactivated at 56°C for 30 min. Streptomycin (100 mg/ml), penicillin G (100 U/ml), and sodium bicarbonate (3.7 g/L) were added to the RPMI 1640 medium before culture. The cultures were prepared using either 6×10^6 or 10^5 cells in 10×30 mm dishes or 24-well tissue culture plates (Costar, Cambridge, MA, USA), respectively.

Cell lines

The Calo cell line was derived from a human cervical carcinoma stage IIB. These are adherent cells with a high proliferation rate. The cells were grown in RPMI 1640 supplemented with 10% (V/V) FBS. L-929 cells are fibroblasts of murine origin and producers of several cytokines, such as M-CSF. L-929 cells were cultured in Iscove4s medium (Gibco, USA) supplemented with 10% (V/V) FBS.

BMCs

To prepare BMCs, RPMI 1640 was flushed through individual mouse femoral shafts using a syringe until the bone marrow tissue was obtained. The cells were pooled and washed three times by centrifugation in RPMI1640 medium, and then resuspended in 10 ml of RPMI 1640 containing 10% (V/V) FBS.

Harvest of band cells

Collected BMCs were centrifuged in a density cushion of ficoll (1.077 gr/ml) for 30 min at $500 \times g$. The cell pellet was washed twice by centrifugation in phosphate buffered saline (PBS), then centrifuged over a ficoll cushion (1.083 gr/ml) for 20 min at $500 \times g$. The cells collected at the interface, mainly band cells, were harvested and washed three times in PBS for subsequent identification using an optical microscope, and cultured as indicated above. In our experimental conditions we obtained 85% band cells, 10% mature polymorphonuclear cells, 3% of macrophages and 2% lymphocytes.

Colony forming assay

For the colony-forming assay, the double agar layer technique was employed to culture BMCs.[21] Briefly, a first layer with 0.6% (W/V) agar was added with RPMI 1640 medium, 20% (V/V) horse serum (HS) and the colony stimulating factor (CSF) or inducer to be tested: 20 ng/ml rhG-CSF or 20 ng/ml rmM-CSF; a second layer with 0.3% (W/V) agar, RPMI 1640 medium and 10% HS (V/V) was overlayered with 10^5 BMCs per well. After 7 days of incubation, all the colonies with more than 20 cells were counted using an inverted microscope.

Production of supernatants and cell lysates

Band cells stimulated with 20 ng/ml of rhG-CSF for 24 and 48 h were collected, as well as their supernatants. The cells were washed twice by centrifugation at $500 \times g$ with

RPMI 1640 medium, and lysed by a double cycle of freeze-thaw using liquid nitrogen. RPMI 1640 was added to the lysate to reach a final concentration of 10^6 lysed cells per millilitre. Supernatants and cell lysates were stored at -20°C until use.

Colony and cell morphology

Evaluation of the morphology of the colonies in the double-layer agar was performed by a modification of a transfer technique on coverslips.[22] Briefly, the upper agar layer was cut into approximately 2.5×1 cm pieces, and a sheet of dry Whatman No. 2 filter paper (Whatman Incorporated, Clifton, New Jersey, USA) of the same size overlaid. As soon as the filter became moist, it was carefully removed. The attached colonies were transferred by placing the filter on a coverslip for 45 min at room temperature. The filter paper was then carefully removed. A smear of band cells from cultures maintained during 24 or 48 h with or without rhG-CSF, as well as the colonies on the coverslip were fixed and stained with Giemsa.

When the colonies consisted of small cells with ring-shaped nuclei or when the nuclei were segmented, they were scored as mature granulocytic colonies; when the cells were large in size and large vacuoles were evident, they were scored as macrophages. Finally, colonies with undifferentiated cells were scored as blastic.

Chromophore labelling of antibodies anti-M-CSF

The Atf-Bct-NHS label reagent (Boehringer, Mannheim D.F. Mexico) was dissolved in DMSO (dimethyl sulfoxide) to a final concentration of 1 mg/100 µl. A solution of 1 µg/ml of anti-M-CSF antibody was prepared, to which 1 µl of Atf-Bct-NHS was added per 9 µl of antibody solution. All this process was performed under dimmed light. Antibodies and chromophore were incubated for 2 h at room temperature and constant shaking.

Detection of M-CSF using labelled anti-M-CSF antibodies

Neutrophils (3×10^6) from cultures treated with or without G-CSF were collected and washed twice in PBS. The cells were resuspended in 90 µl of RPMI, and 10 µl of chromophore-labelled antibodies were added and incubated in the dark for 20 min at 37°C.

Cells were washed twice in PBS to eliminate the excess labelled antibodies, 100 µl of the cells were used to prepare slides by cytospins (30 µl per slide). The cells were fixed immediately with methanol, dried and evaluated under a microscope using UV light (BH-2, Olympus, Japan).

ELISA

The primary antibody was a goat-anti-mouse IgG anti-M-CSF, and the secondary was a peroxidase-labelled rabbit-anti-goat antibody. The presence of M-CSF was tested in ELISA, according to the method described by Kemeny.[23] Briefly, 100 µl/well of conditioned medium or 12 ng of rmM-CSF in borate buffer (pH 9.5) was added to 96-well immunoplates (Costar, USA). After incubation for 2 h at 37°C, followed by 4°C overnight, the wells were washed with 0.1% (V/V) Tween-20 in PBS (Tween/PBS) and blocked by adding 100 µl/well of 2% (W/V) bovine serum albumin (BSA) in PBS for 1 h at 37°C. The BSA/PBS solution was discarded and 100 µl/well of the corresponding primary antibody (anti-M-CSF, goat anti-mouse IgG) were added, diluted at a concentration of 1:200 in BSA/PBS and incubated for 3 h at 37°C.

After washing with Tween/PBS, the secondary antibody (peroxidase-conjugated rabbit anti-goat), was added at a concentration of 1:3000 in BSA/PBS, and incubated for 2 h at 37°C. Six milligrams of ortho-phenylendiamine were dissolved in 12 ml of citrate buffer (pH 5) and used as substrate for peroxidase; 10 µl of H_2O_2 was added to the solution immediately before use. 100 µl/well were added and the plate kept in darkness for 10 min at room temperature. The reaction was stopped by adding 25 µl/well of H_2SO_4 (1.25 M). The plates were read at 490 nm in an ELISA reader (EL × 800 Bio-tek Instruments, Inc. NY, USA).

Inhibition of colony formation by anti-M-CSF antibodies

In order to confirm that the colony forming activity from band cells treated with G-CSF was due to secretion of M-CSF, anti-M-CSF monoclonal antibodies were added in the colony assay (see above). BMCs were plated in agar and stimulated or not with the supernatant of band cells treated during 48 h with G-CSF. To this assay, anti-M-CSF antibodies were added at a 1:200 dilution in the first agar layer.

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