A Monokine Regulates Colony-Stimulating Activity Production by Vascular Endothelial Cells

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Human umbilical vein endothelial cells were cultured in supernatants of peripheral blood monocytes that had been cultured for 3 days with and without lactoferrin. Colony-stimulating activity (CSA) was measured in supernatants of the endothelial cell cultures and appropriate control cultures using normal, T-lymphocyte-depleted, phagocyte-depleted, low-density bone marrow cells in colony growth (CFU-GM) assays. Monocyte-conditioned medium contained a nondialyzable, heat labile factor that enhanced 4-15-fold the production of CSA by endothelial cells. The addition of lactoferrin to monocyte cultures reduced the activity of this monokine by 69%. Lactoferrin did not inhibit CSA production by monokine-stimulated endothelial cells. Therefore, vascular endothelial cells are potent sources of CSA, the production of CSA by these cells is regulated by a stimulatory monokine, and the production of CSA is inhibited by lactoferrin, a neutrophil-derived putative feedback inhibitor of granulopoiesis. Inasmuch as a similar monokine is known to stimulate CSA production by fibroblasts and T lymphocytes, we suggest that mononuclear phagocytes play a pivotal role in the regulation of granulopoiesis by recruiting a variety of cell types to produce CSA.

Granulocytes and mononuclear phagocytes are descendants of hemopoietic progenitor cells (CFU-GM) that form granulocyte and macrophage colonies in semisolid media. The clonal growth of these progenitor cells depends on a family of regulatory glycoproteins known as colony-stimulating activity (CSA). CSA is produced by a variety of cell types, including mononuclear phagocytes, T lymphocytes, fibroblasts, and vascular endothelial cells, all of which are essential elements of the hemopoietic marrow and its microenvironment. We hypothesize that if CSA is a biologically relevant granulopoietic stimulator, its production by these heterogeneous cell types should be in some way integrated and subject to positive and negative regulatory control. Support for this hypothesis comes from our recent finding that CSA production by T lymphocytes is regulated by a soluble monocyte-derived factor [monocyte-derived-recruiting activity, (MRA)] and that the production or release of this stimulatory monokine is inhibited by lactoferrin, a putative feedback regulator of neutrophil production. We now describe experiments that demonstrate that similar interactions occur between monocytes and endothelial cells.

MATERIALS AND METHODS

Experimental Design

Each study consisted of three steps: first, monocytes were cultured to produce monocyte-conditioned medium (MCM); second, the MCM (which contains MRA) and appropriate control media were incubated with cultures of endothelial cells; third, the CSA content of endothelial cell supernatants was measured.

Monocyte Cultures

Monocytes were isolated from E-rosette-depleted peripheral blood mononuclear leukocytes of normal volunteers by adherence to serum-coated dishes. The adherent monocytes, which were more than 90% alpha-naphthyl butyrate esterase positive, were lifted off the dishes by EDTA treatment (0.2%, 4°C, 20 min), washed twice, and cultured in 60-mm culture dishes at a concentration of 2–3 x 10⁵ cells/ml in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 mM glutamine, 15% lactoferrin-depleted fetal calf serum (FCS), and antibiotics for 3–5 days at 37°C in a humidified incubator (7.5% CO₂, air). Dishes containing culture medium without monocytes served as controls. At the end of the incubation period, the monocyte-conditioned medium and the control medium were harvested, diluted 1:1 with RPMI 1640, and added to cultures of endothelial cells (see below). In two experiments, monocytes were treated with OKT3 and OKT3 with complement, washed, and then plated in 3-day cultures.

In another series of experiments, monocytes were cultured in the presence of lactoferrin. In these experiments, lactoferrin, at a final concentration of 10⁻⁹M, was added to the monocytes at the start of the incubation period.

Treatment of MCM

In further experiments designed to investigate the properties of MRA, MCM was subjected to a variety of treatments before its addition to the endothelial cell cultures. These treatments included repeated freezing to −20°C, with rapid thawing to room temperature, refrigeration for 4 days (4°C), heating to 56°C for 30 min, 80°C for 5 min, and 100°C for 2 min, and dialysis against phosphate-buffered saline (PBS) at 4°C (3 days, 4 exchanges, 10–12,000 dalton cutoff).

Endothelial Cell Cultures

Human umbilical vein endothelial cells were prepared according to previously described techniques by limited collagenase (Worthington, Type 1, 0.1% w/v in phosphate-buffered saline) treatment. The cells were seeded into 16-mm tissue culture wells and cultured in...
RPMM 1640 or medium 199 (Microbiological Associates, Walkersville, MD), supplemented with 25 mm HEPES buffer, 2 mm glutamine, 20% fetal calf serum, antibiotics, and 250 μg/ml endothelial cell growth factor, at 37°C in a humidified incubator (95% air 5% CO₂). Cells were subcultured by brief (5 min, 37°C) treatment with 0.02% EDTA/0.05% trypsin (GIBCO) and were reseeded in 16-mm wells coated with fibronectin (2 μg/sq cm; Collaborative Research, Lexington, MA). Both primary and subcultured cells exhibited the cobblestone morphology characteristic of endothelium; the endothelial nature of the cells was further confirmed by the fluorescent perinuclear granules visible in more than 90% (in some cases 98%) of the cells after staining with rabbit anti-human factor-VIII-related antigen and a fluorescent-labeled second antibody (goat anti-rabbit IgG, Cappel Laboratories, Cochranville, PA). Cyt fluorographic analysis of multiply passaged endothelial cells exposed to monoclonal antibodies with reactivity to antigen markers characteristic of mononuclear phagocytes (OK-MI, Mac-120) and T lymphocytes (OKT3, OKT4) showed no contamination of endothelial cell cultures by these cell types.

At least 24 hr were allowed to elapse after initial seeding of primary cultures or subcultures of passaged cells before the MCM was added to the cultures. The endothelial cell culture medium was removed and replaced with diluted MCM or control medium. The endothelial cells were then returned to the incubator for a further 3-5 days before the supernatants were removed, centrifuged (200 g, 5 min), and assayed for CSA content. In two experiments, subconfluent cultures of endothelial cells in the 14th-16th passage were radiated with 2,000 R prior to culture in MCM. After a 6-hr culture in MCM, CSA production and ³H-TdR incorporation (1 μCi/ml, 5 Ci/m mole, 1 hr pulse) were measured in these and unirradiated control cells.

**CSA Assay**

CFU-GM colony growth of low-density (Ficoll-Hypaque, Pharmacia Fine Chemicals, Piscataway, NJ), macrophage-depleted (iron/magnet), E-rosette-depleted marrow cells served as our CSA assay. For each sample, 10⁵ marrow cells were cultured in 1 ml 0.9% methylcellulose in alpha medium supplemented with 15% FCS. The methylcellulose was layered an agar base (0.5% w/v in McCoy's 5a medium) to which had been added 10% v/v of the sample to be assayed. The negative control contained 10% medium only. HPCM prepared as previously described served as a positive control. Colonies (aggregates >39 cells) and clusters (<40, >8 cells per aggregate) were counted on days 7-10 of culture.

**RESULTS**

**CSA Levels in Endothelial Cell Supernatants**

Supernatants of endothelial cells cultured for 3-5 days in monocyte-conditioned medium (ECM₅₀) contained more CSA than did either MCM incubated in the absence of endothelial cells, or supernatants of endothelial cells cultured for the same period of time in unconditioned medium (ECM). In 14 experiments (Fig. 1), colony growth stimulated by ECM₅₀ was 4-15 times greater than that stimulated by ECM plus MCM, an increase that was significant (p < 0.01, Student's t test). MCM produced by OKT3-treated monocytes (with and without complement) was as active as untreated MCM (data not shown). We therefore attribute this stimulatory effect of monocyte-conditioned medium to the presence in it of the soluble monokine, MRA. We have previously shown that a soluble monokine present in MCM stimulates CSA production by T lymphocytes. Accordingly, we carried out experiments designed to rule out a potential effect of MRA on T cells that may have contaminated the endothelial cell cultures. Depletion of E-rosette-positive cells in the endothelial cell suspensions prior to culture did not alter the effect of MRA on CSA production by endothelial cells.

The effect of MRA required intact endothelial cells: mixtures of endothelial-cell-conditioned medium (ECM) and MCM contained no more CSA than ECM or MCM alone (2 experiments). In addition, the effects of MRA in primary (138 ± 31 colonies/10⁵ cells, n = 6 experiments), confluent primary (120 ± 40, n = 2), and multiply passaged cultures (130 ± 50, n = 11) were similar. In 2 experiments, MCM enhanced ³H-TdR incorporation by endothelial cells by 3-4-fold. Radiation (2,000 R) of endothelial cells abrogated the effect of MCM on ³H-TdR incorporation, but did not inhibit CSA production in response to MCM (data not shown).

Endothelial cells did not produce MRA-like factors. Specifically, in each of 13 experiments, endothelial cell
conditioned medium (0.5–5.0 × 10⁵ cells/ml in RPMI 1640 supplemented with 20% FCS and antibiotics) failed to enhance or stimulate CSA production by either T lymphocytes (0.5–2.5 × 10⁵ cells/ml) or peripheral blood monocytes (2–5 × 10⁵ cells/ml). T-lymphocyte-conditioned medium also failed to stimulate CSA production by endothelial cells (data not shown).

**Limiting Dilution Studies**

In 4 experiments, monocytes were resuspended at different concentrations prior to incubation, and the resulting MCMs were multiply diluted and added to endothelial cell cultures. The CSA content of the ECMₖ derived in one of these experiments is shown in Fig. 2. As can be seen, endothelial-cell-derived CSA increased as the monocyte concentration used to prepare the MCM increased. As few as 1,000 monocytes/ml were able to condition medium adequately to effect an increase in the CSA content of the ECMₖ (data not shown). Even with 10⁴ and 10⁵ monocytes/ml, the stimulating effect on endothelial cells was detectable in MCM even when monocyte-derived CSA was not (Fig. 2). The optimal monocyte concentration for provision of MRA appeared to be 10⁵/ml, and the effective concentration of MCM for endothelial cell cultures ranged from 20% to 50%.

In 4 experiments, passaged endothelial cells were seeded at concentrations ranging from 10⁵/well to 10⁶/well, and their CSA production was determined in response to a 50% dilution of MCM prepared from 10⁵ monocytes/ml tested. In 3 of 4 experiments, as few as 10⁴ endothelial cells were measurably responsive to MCM, and increasing levels of CSA were found with increasing cell numbers (Fig. 3).

**Effect of Lactoferrin**

Lactoferrin (concentration 10⁻¹⁴M) was added to monocyte cultures prior to the incubation period. The resulting conditioned medium was harvested and added to endothelial cell cultures. CSA content of the endothelial supernatants in this experiment (ECM₃₅₇) was compared to that of endothelial cells cultured in MCM prepared without lactoferrin (EC₃₇). In 9 of 12 experiments, the CSA in ECM₃₅₇ was significantly reduced (p < 0.001, Student’s t test) compared with that of ECM₃₇. The mean (±SD) percent reduction in these 9 experiments was 69% ± 24%. In 3 of 12 experiments, lactoferrin did not diminish MRA production.

**Preliminary Characterization of MRA**

As shown in Fig. 4, MRA was stable in storage at 4°C, but was lost with repeated freeze–thawing. Activity was nondialyzable and stable after heating at 56°C for 30 min, but heating at 80°C for 5 min or 100°C for 2 min resulted in a loss of activity.

**DISCUSSION**

A number of groups have reported that mononuclear phagocytes are potent sources of CSA.⁴ ⁵ This notion derived from in vitro studies that failed to remove MRA-responsive cells (i.e., T lymphocytes) from either the monocytes that produced the CSA or the bone marrow cells in the CSA assays. Although the low CSA titers in our MCM derive in part from our

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**CSA ASSAYS**

![Fig. 2. Limiting monocyte dilution curves. The results shown are those of one of four similar experiments. Multiple concentrations of 3 different MCMs (made by 10⁴, 10⁵, and 10⁶ monocytes/ml) were added to endothelial-cell cultures. CSA titer is expressed as mean colonies ± SD. Colony growth stimulated by each MCM alone is represented by the dose–response curves labeled M. Colony growth stimulated by endothelial-cell-conditioned medium is represented by the dose–response curves labeled E.](image)
purposeful selection of a day-3 harvest date (day-7 MCM contains more CSA), we have also noted that removal of MRA-responsive T lymphocytes causes a significant reduction of CSA in MCM. We are, therefore, unimpressed with the unstimulated monocyte as a potent CSA producer. However, we have noted that monocytes do produce factors, MRA, which stimulate other cells to produce CSA and that the production of MRA is inhibited by lactoferrin. In this study, we document that vascular endothelial cells also respond to MRA. The ECGF-dependency of our endothelial cells and their factor VIII positivity (91%–98%) indicate that the responder cells were, indeed, endothelial rather than another MRA-responsive cell type. Even if the 2%–9% factor-VIII-negative cells represented another MRA-responsive cell type, fibroblasts for example, the limiting dilution study rules them out as the MRA-responsive cell in this study. We noted that 1,000 endothelial cells/ml responded to MRA. Nine percent contamination by fibroblasts would not have provided enough fibroblasts to respond to MRA (1,000/ml are required). We also noted that endothelial cells do not produce MRA-like factors. Thus, as we have shown in other studies, the monocyte is the only cell of the four types we have studied that is capable of recruiting other cells to produce CSA.

Few of the monokines have been characterized biochemically, and several biologic activities in monocyte-conditioned medium may be attributable to a
single molecule. Thus, we do not know whether the MRA that stimulates endothelial cells is a heretofore undescribed factor or an additional activity of a described factor. Although MRA may represent the monokine that stimulates endothelial cell proliferation in vitro, that our monocyte supernatants were inactivated upon freeze/thawing (the proliferation factor is active after such treatment), suggests otherwise. At least endothelial cell proliferation is not required for monokine-induced CSA production, because MCM actively stimulated CSA production by endothelial cells that had been rendered nonproliferative by radiation in vitro. MRA could also represent the same factor that stimulates CSA production by T lymphocytes and fibroblasts. In addition, MRA may, like CSA, represent a family of molecules with differing target cell specificities and/or affinities. In this regard, it is of interest to note that while lactoferrin-mediated inhibition of MRA production ranges from 75%–100% when T cells or fibroblasts are used as targets, inhibition of MRA in the endothelial cell assays is only 66% ± 24% (range 35%–100%). In fact, in 3 of 12 experiments, LF did not reduce MRA in MCM. Further studies will be required to determine the mechanisms that underlie the apparent heterogeneity of lactoferrin responsiveness among various target cells.

In these studies, we have avoided mixing monocytes with endothelial cells in culture because such cocultures might, because of antigenic nonidentity, result in augmentation of CSA production, a phenomenon that has been described in the allogeneic mixed leukocyte reaction. It is also theoretically possible that antigenic determinants may be present in cell products released into culture medium and that the effect of MCM on endothelial cells reflects an interaction of such an antigen (MRA) with endothelial cells. We do not believe this to be the case, because we have found that allogeneic and autologous T lymphocytes and marrow-derived reticular cells (unpublished) are equally responsive to MRA.

We noted, but do not yet understand the reasons for, increased MRA activity after storage at 4°C or after heating (Fig. 3). Mononuclear phagocytes are known to secrete a variety of biologically active factors, including hydrolytic enzymes, oxygen metabolites, complement components, arachidonic acid oxygenation products, and numerous factors (monokines) that regulate the behavior of other somatic cells. Many of these factors, which could themselves have been inactivated by these treatments, may have inhibited the activity of MRA, inhibited the production of CSA by endothelial cells, inactivated CSA, or directly inhibited colony growth (e.g., prostaglandin E2).

CSA is widely regarded as a likely candidate for a biologically relevant "granulopoietin," but its role in regulating granulopoiesis in vivo has not been established. Studies on the biologic relevance of CSA under steady-state conditions are fraught with conceptual pitfalls. For example, most of the cell types known to produce CSA do so principally when stimulated by exogenous factors, such as mitogens, endotoxin, or antigens, conditions that fail to approximate the steady state. It has also been conceptually difficult to accept a major regulatory role for a factor produced independently by such diverse cell types. The results of our studies indicate that CSA production by a variety of cell types does not occur independently, but is under the regulatory control of mononuclear phagocytes. We suggest that the mononuclear phagocyte is a pivotal granulopoietic regulatory cell, but that it plays its major role, not by producing CSA, but by stimulating other cells to produce CSA.

REFERENCES

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GC Jr Bagby, E McCall, KA Bergstrom and D Burger