

# Regulation of Colony-stimulating Activity Production

## INTERACTIONS OF FIBROBLASTS, MONONUCLEAR PHAGOCYTES, AND LACTOFERRIN

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**ABSTRACT** Neonatal skin fibroblasts were cultured in supernatants of peripheral blood monocytes that had been cultured with and without lactoferrin. Granulocyte-monocyte colony-stimulating activity (CSA) was measured in supernatants of the fibroblast cultures with normal T lymphocyte-depleted, phagocyte-depleted, low density bone marrow target cells in colony growth (colony-forming unit granulocyte/macrophage) assays. Monocyte-conditioned medium contained a nondialyzable factor that enhanced by 17–50-fold the production of CSA by fibroblasts. The addition of lactoferrin to monocyte cultures reduced the activity of this monokine by 75–100%. Lactoferrin did not inhibit CSA production by monokine-stimulated fibroblasts. We conclude that under appropriate conditions human fibroblasts are potent sources of CSA, that the production of CSA by these cells is regulated by a stimulatory monokine, and that the production and/or release of the monokine is inhibited by lactoferrin, a neutrophil-derived putative feedback inhibitor of granulopoiesis. We propose that the major role of mononuclear phagocytes in granulopoiesis is played not by producing CSA, but by recruiting other cells to do so, and that in the steady state, feedback regulation of neutrophil production may occur as a result of a mechanism that inhibits the recruitment phenomenon.

### INTRODUCTION

The production of neutrophils and mononuclear phagocytes in clonal cultures of human bone marrow cells is dependent upon a family of regulatory gly-

coproteins known as granulocyte-monocyte colony-stimulating activity (CSA)<sup>1</sup> (1–3). CSA is produced by a variety of normal cell types including mononuclear phagocytes (4–6), T lymphocytes (6–8), and vascular endothelial cells (9, 10).<sup>2</sup> Murine fibroblast cell-lines produce CSA (11), but recent reports suggest that neither human nor murine bone marrow-derived (12–15) or skin-derived (15) fibroblasts produce CSA in culture. We have recently described a soluble factor produced by mononuclear phagocytes that stimulates CSA production by T lymphocytes (6) and endothelial cells.<sup>2</sup> The latter two cell types produce little CSA in culture unless they are stimulated with exogenous factors such as mitogens (6, 7), endotoxin (10), or a monokine (6).<sup>2</sup> The potential physiological significance of the monokine is further supported by our observation that its production or release is inhibited by low concentrations of lactoferrin (6), a putative feedback regulator of granulopoiesis (16, 17). We now describe experiments that demonstrate that skin-derived human fibroblasts can produce CSA when stimulated by a soluble factor released by mononuclear phagocytes, and that the production and/or release of the regulatory monokine is inhibited by lactoferrin.

<sup>1</sup> *Abbreviations used in this paper:* CSA, granulocyte-monocyte colony-stimulating activity; FCM, fibroblast-conditioned medium; FCM<sub>M</sub>, conditioned medium from fibroblasts cultured in MCM; FCM<sub>MLF</sub>, conditioned medium from fibroblasts cultured in conditioned medium from monocytes cultured in medium containing 10<sup>-12</sup> M lactoferrin; FCM<sub>LF</sub>, conditioned medium from fibroblasts cultured in MCM to which had been added (after monocyte supernatants were harvested) 10<sup>-12</sup> M lactoferrin; FCS, fetal calf serum; HPCM, human placental-conditioned medium; MCM, monocyte-conditioned medium.

<sup>2</sup> Bagby, G. C., K. Bergstrom, and E. McCall. 1982. Submitted for publication.

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## METHODS

**Fibroblast cultures.** Human skin fibroblasts obtained from explants of neonatal foreskin were prepared according to previously described techniques (18). Explants were cultured in McCoy's 5A medium (Flow Laboratories, Inc., Rockville, MD) containing 20% human serum and antibiotics. Primary outgrowths of cells were dissociated with 0.02% EDTA-trypsin, and subcultured in 60-mm culture dishes (Falcon Manufacturing of Michigan, Inc., Byron Center, MI) containing McCoy's 5A medium with antibiotics in 10% pooled human AB serum. No erythrocyte (E) rosette-positive cells were detectable in the explanted fibroblasts. These cells did not contain material reactive with a Factor VIII antibody,<sup>2</sup> and were OKMI (Orthoclone, Ortho Pharmaceutical Corp., Raritan, NJ) negative. Confluent cultures were incubated for 24 h in McCoy's 5A medium lacking unlabeled proline and glycine and containing tritiated proline and glycine (10  $\mu$ Ci/ml), 10% human serum, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin. The cell layer was scraped from the flask with the medium, pooled, and lyophilized. The lyophilized samples along with carrier collagen were digested with CNBr in 70% formic acid. The resulting radioactive peptides were separated by chromatography as previously described (19). These cells produced 85–90% type I and 10–15% type III collagen. Collagen represented 15% of the total protein produced by these cells in vitro.

All experiments were performed by using cells between the third and eighth subculture, with the cells in the late logarithmic phase of growth. The doubling time of these cells ranged from 22 to 24 h. Cells were incubated either in RPMI 1640 with 15% lactoferrin-depleted fetal calf serum (FCS) or in a variety of monocyte-conditioned media (MCM) at 37°C for 3 d. The resulting fibroblast-conditioned media (FCM) were tested for CSA in the granulocyte colony-growth assay described below.

**Mononuclear phagocytes.** Monocytes were isolated from peripheral blood mononuclear leukocytes of normal volunteers as previously described (6) by adherence to serum-coated dishes. The adherent cells, which were detached from the dishes with 0.02% EDTA, were >90% alpha naphthyl butyrate esterase-positive. These monocytes were resuspended at a concentration of  $2-3 \times 10^5$ /ml in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 15% lactoferrin-depleted FCS, and incubated for 3–5 d at 37°C in a 7.5% CO<sub>2</sub> atmosphere. Monocytes were also cultured in the presence of  $10^{-12}$  M human breast-milk lactoferrin as previously described (6, 18). The MCM harvested on the final day of culture was diluted 1:1 with complete RPMI medium and was used to replace the fibroblast culture medium (see above). The supernatants of fibroblasts cultured with MCM (FCM<sub>M</sub>) and without MCM (FCM) were harvested after incubation at 37°C for 3 d and tested for CSA at concentrations of 5 and 10% in the colony growth assay. In two experiments, monocytes ranging from  $10^2$ /ml to  $10^6$ /ml were used in culture to generate conditioned media that were harvested after 3 d of culture for CSA assays and fibroblast cultures. Conditioned medium (50%) from monocytes cultured in  $10^{-12}$  M lactoferrin was also used in three fibroblast culture experiments. The activity of the resultant FCM<sub>MLF</sub> was compared with that of conditioned medium of fibroblasts cultured in MCM to which lactoferrin ( $10^{-12}$  M) had been added. MCM controls were diluted 1:1 with complete medium, incubated for 3 d on plastic dishes in parallel with fibroblast cultures, and tested for CSA as were FCM samples.

Other conditioned media assayed for CSA included dialyzed MCM and MCM prepared with monocytes cultured in medium with  $10^{-7}$  M indomethacin (Sigma Chemical Co., St. Louis, MO). MCM was assayed for colony inhibitory activity by adding 10% MCM to conditioned medium from peripheral blood mononuclear leukocytes stimulated with 1% phytohemagglutinin (10%)-stimulated and to human placental-conditioned medium (HPCM) (10%)-stimulated cultures of marrow cells as described below.

**Granulocyte colony growth assay.** Colony-forming unit granulocyte/macrophage colony growth of low density (Ficoll-Paque, Pharmacia Fine Chemicals Div. of Pharmacia Inc., Piscataway, NJ) macrophage-depleted (iron/magnet) E rosette-depleted marrow cells (6, 17)<sup>2</sup> served as our CSA assay. Assays were performed with marrow cells from five paid normal adult volunteers in five separate experiments. Sources of CSA were 10% FCM, MCM, FCM<sub>M</sub>, FCM<sub>MLF</sub>, conditioned medium from fibroblasts cultured in MCM to which had been added (after monocyte supernatants were harvested)  $10^{-12}$  M lactoferrin (FCMLF<sub>M</sub>), and HPCM prepared as previously described (20). In each experiment, exogenous CSA was required for colony growth (no "spontaneous" colony growth was noted). Marrow cells ( $10^5$ ) were cultured in 1 ml 0.9% methylcellulose in alpha medium supplemented with 15% lactoferrin-depleted FCS (6). The methylcellulose was layered over agar (0.5%) medium containing CSA and/or other test materials. Colonies (aggregates >39 cells) and clusters (<40, >8 cells/aggregate) were counted after 7 d of culture. Differences between colony scores (three to five replicate plates) were considered significant if the *P* value was <0.05 with the *t* test.

**Treatment of MCM.** MCM was tested freshly harvested, after incubation for 3 d, heating to 56°C for 30 min, and dialysis for 3 d (four exchanges) against phosphate-buffered saline (PBS) at 4°C with a dialysis membrane with a mol wt cutoff of 12,000.<sup>2</sup>

## RESULTS

In each of four experiments there was more than a 17-fold CSA increase in FCM<sub>M</sub> compared with MCM plus FCM. CSA of FCM<sub>MLF</sub> was significantly reduced (*P* < 0.001) compared with that of FCM<sub>M</sub>. However, in FCMLF<sub>M</sub>, CSA was unchanged compared with FCM<sub>M</sub> (Fig. 1). Colony growth stimulated by FCM<sub>M</sub> vs. MCM or FCM was significantly different (*P* < 0.001) in all experiments (*t* tests), and mean colony growth/ $10^5$  cells for the group of experiments was significantly greater when cultures were stimulated by FCM<sub>M</sub> (*P* < 0.001). Thus, supernatants of fibroblasts cultured for 3 d in MCM contained more CSA than supernatants of fibroblasts or monocytes cultured in medium alone (Fig. 1). This effect of MCM required intact fibroblasts because mixtures of FCM and MCM contained no more CSA than FCM or MCM alone (data not shown). The stimulatory activity in MCM was readily detectable in the 3-d supernatant medium of  $10^3$  monocytes/ml that was devoid of CSA (Table I).

The monocyte/fibroblast interaction is unidirectional: FCM (supernatants of 3-d cultures as above)

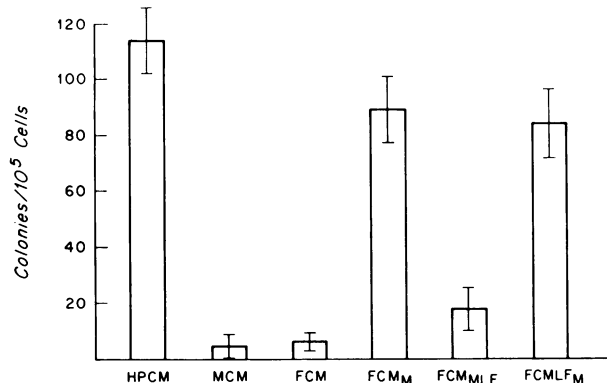


FIGURE 1 The effect of MCM on CSA production by fibroblasts. Bars and vertical lines represent means $\pm$ SD. CSA production is represented on the Y axis as colonies per 10<sup>5</sup> cells. Target cells were depleted of phagocytes and T lymphocytes. No colony or cluster growth was seen in any plate unless active conditioned media were added. These data were obtained in one of five similar experiments using five separate sources of MCM, and five separate sources of normal bone marrow cells. The sources of CSA were HPCM, MCM, FCM, FCM<sub>M</sub>, FCM<sub>MLF</sub>, and FCMLF<sub>M</sub>.

consistently failed to enhance or stimulate CSA production by monocytes (2–5  $\times$  10<sup>5</sup> monocytes/ml incubated for 3 d in 10, 50, and 100% FCM), and endothelial cell-conditioned media and T lymphocyte-conditioned media failed to stimulate CSA production by fibroblasts (data not shown). Thus, the monocyte seems to be the only CSA-producing cell type of the four we have studied (monocytes, T lymphocytes (6), endothelial cells,<sup>2</sup> and fibroblasts), that is capable of stimulating other cells to produce CSA.

The increased levels of CSA present in FCM<sub>M</sub> compared with those found in MCM could have been the result of an inhibitor in MCM that masked CSA in

MCM and was somehow lost during incubation with fibroblasts. Experiments were performed to examine this possibility. We found that 10% MCM did not inhibit colony growth of maximally stimulated marrow cells (140 $\pm$ 16 colonies/10<sup>5</sup> cells [HPCM], 136 $\pm$ 12 [HPCM + MCM]). No inhibitory activity was found when conditioned medium from peripheral blood mononuclear leukocytes stimulated with 1% phytohemagglutinin was used instead of HPCM. In addition, dialysis of MCM failed to enhance CSA in MCM. Finally, indomethacin failed to augment CSA in MCM, and also failed to increase CSA in FCM<sub>M</sub> (FCM<sub>M</sub> = 88 $\pm$ 13 colonies/10<sup>5</sup> cells, FCM<sub>M</sub>-indomethacin = 98 $\pm$ 19 colonies/10<sup>5</sup> cells). Preliminary characterization of the monocyte-derived fibroblast stimulatory activity has shown that it is nondialyzable and stable after 4 d at 4°C and relatively stable after heating to 56°C for 20 min (Fig. 2).

## DISCUSSION

Recent studies using long-term marrow cell culture techniques (21) have documented that fibroblasts derived from murine marrow augment survival of hemopoietic stem cells (13, 14). However, although murine fibroblast cell-lines produce CSA (11), a number of groups have failed to detect CSA in conditioned media of either murine (13, 14) or human (12, 15) marrow or skin fibroblast cultures. The results of our study with cultures of neonatal skin fibroblasts support the view that the production of CSA by unstimulated fibroblasts is negligible.

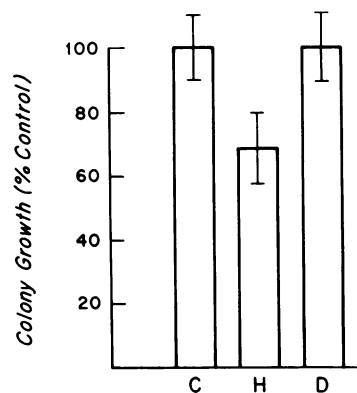


FIGURE 2 The effect of various treatments on the activity in MCM that stimulates CSA production by fibroblasts. MCM was treated before fibroblast cultures. MCM was tested untreated (C), after heating to 56°C for 30 min (H) and after dialysis against PBS (D) at 4°C (four exchanges in 3 d). Bars and vertical lines represent means $\pm$ SD (expressed as percentage control where control is C) of two separate experiments. Mean CSA content of FCM<sub>M</sub> decreased by 32% when heated MCM (H) was used in fibroblast cultures.

TABLE I  
Influence of Monocyte Numbers on Monokine  
Content of MCM\*

Monocytes/ml	MCM		FCM <sub>M</sub>	
	Colonies	Clusters	Colonies	Clusters
10 <sup>2</sup>	0	0	0	13 $\pm$ 4
10 <sup>3</sup>	0	3 $\pm$ 1	128 $\pm$ 8	88 $\pm$ 8
10 <sup>4</sup>	0	4 $\pm$ 2	96 $\pm$ 18	90 $\pm$ 12
10 <sup>5</sup>	0	7 $\pm$ 2	176 $\pm$ 24	112 $\pm$ 16
10 <sup>6</sup>	0	4 $\pm$ 2	89 $\pm$ 6	80 $\pm$ 16

\* Monocyte supernatants were removed on the third day of culture, diluted 1:5 with complete medium, and then either tested for CSA (MCM) or placed on fibroblast monolayers for 3 d after which the fibroblast supernatant (FCM<sub>M</sub>) was harvested and tested for [CSA].

We had previously demonstrated that soluble factors released from mononuclear phagocytes stimulate T lymphocytes and endothelial cells (6)<sup>2</sup> to produce large amounts of CSA. Moreover, mononuclear phagocytes are known to facilitate proliferation of both fibroblasts (22, 23) and endothelial cells (23). Accordingly, we proposed that fibroblasts, like T lymphocytes and endothelial cells, would be stimulated to produce CSA by a factor released by mononuclear phagocytes. In this study we did find that human MCM contains a nondialyzable, heat-stable factor that stimulates fibroblasts to produce CSA (Figs. 1 and 2). That these multiply-passaged, spindle shaped cells synthesized types I and III collagen and that T lymphocytes, endothelial cells, and macrophages were not present in these cultures document that MCM interacted with fibroblasts and not with another monokine-responsive cell population. In addition, the production or release of the monokine was markedly inhibited by lactoferrin, a putative feedback regulator of granulopoiesis (6, 16–18)<sup>2</sup> (Fig. 1).

Because CSA measurements involve a colony growth assay, there exists a potential for error when antipodal activities are present in a test medium. Thus, it was possible that potent CSA was present in MCM but masked by an inhibitor of colony growth (e.g., prostaglandin E [24]) which was lost during the fibroblast cultures. Experiments that effectively ruled out this possibility showed that 10% MCM did not inhibit colony growth of maximally stimulated marrow cells. In addition, dialysis of MCM failed to enhance CSA in MCM. Finally, indomethacin, at doses known to abrogate prostaglandin E synthesis *in vitro* (23), failed to augment CSA in MCM or in FCM<sub>M</sub>. We conclude that there was no inhibitor of clonal growth in MCM that could have accounted for the greater CSA observed in FCM<sub>M</sub> and that fibroblasts are indeed potent sources of CSA.

Few of the monokines have been characterized biochemically and several biological activities in MCM may represent functions of a single molecule. At this time, we do not know whether the activity observed in our experiments represents a novel monokine or simply an additional activity of a previously described factor. This monokine could, for example, represent the same factor that stimulates CSA production by T lymphocytes (6) and endothelial cells.<sup>2</sup> Indeed, there exist some similarities; lactoferrin inhibits the production of the monokine that recruits T lymphocytes (6) and the monokine that stimulates CSA production by endothelial cells is heat stable and nondialyzable.

It is possible that this monokine represents an inactive “pro” CSA molecule that fibroblasts (and other cells) activate. However, we do not believe that this

activity represents the same factor that stimulates fibroblast proliferation *in vitro* (21) because in our experiments, MCM failed to stimulate proliferation of fibroblasts. Furthermore, the activity of MCM in confluent fibroblast cultures suggests that target cell proliferation is not required for the CSA-enhancing effect of MCM.

CSA has been widely regarded as a likely candidate for a biologically relevant “granulopoietin” (25, 26) but its role in regulating granulopoiesis *in vivo* has not been established. Studies on the biological 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 (7, 8) or endotoxin (10), conditions that fail to approximate the steady state. Although it seems reasonable to propose that regulatory cell diversity confers upon a species an evolutionary survival advantage, it has been difficult to accept a major regulatory role for a factor produced independently by such diverse cell types most of which are consistently unresponsive *in vitro* to neutrophil-derived feedback signals (10, 16). Although our studies have confirmed the diversity of CSA-producing cell types, we have also demonstrated that the production of CSA by these cells does not occur independently, rather it is under the regulatory control of mononuclear phagocytes.

It should be emphasized that the nonfibroblast target cells of these monokines can be stimulated in other ways to produce CSA (e.g., with endotoxin [10] or mitogens [6, 7]) and in those settings lactoferrin fails to inhibit CSA production (10, 16). Inasmuch as these stimuli *in vivo* result in an increase in neutrophil production, the responsiveness of the CSA-producing cell population to the stimulatory substance would be theoretically expected as would their refractoriness to a neutrophil-derived inhibitor of steady-state granulopoiesis. Therefore, if the lactoferrin-inhibited monokine proves to have *in vivo* relevance, it may be in regulating granulopoiesis specifically in the steady state where CSA production should be responsive to neutrophil-derived feedback inhibition. It is for this reason that we have assiduously avoided stimulating our monocytes or other cells with exogenous factors (e.g., endotoxin) that would perturb the steady state. A number of investigators have reported that mononuclear phagocytes are potent sources of CSA (4–5). The low CSA titer in our MCM derives, in part, from our selection of a day 3 harvest date (monocyte-derived CSA is low at that time). More importantly, in all previous studies, CSA assays have been carried out with target cells that by virtue of their content of T lymphocytes contain cells responsive to a recruiter

monokine as well as CSA. We believe the previous estimates of CSA in MCM are spuriously high and suggest that future CSA assays be performed with target cells depleted not only of adherent or phagocytic cells, but of T lymphocytes as well.

That CSA is low (6)<sup>2</sup> (Fig. 1) in day 3 supernatants of unstimulated monocytes specifically depleted of auxiliary cells (i.e., cells that produce CSA when stimulated by the monokine), and that the monokine-responsive cells become potent sources of CSA only when stimulated by the monokine, suggest that the major role of mononuclear phagocytes in the regulation of steady-state granulopoiesis is not played by producing CSA, but by recruiting other cells to do so. This point of view is further strengthened by our observations that lactoferrin inhibits the production or release of this monokine but does not directly inhibit CSA production by monocytes or other target cells (6)<sup>2</sup> (Fig. 1). Therefore, although lactoferrin-mediated feedback regulation of neutrophil production in vitro ultimately results in inhibition of CSA production, the effect is an indirect one and results from lactoferrin's inhibition of the recruitment phenomenon. Identification and purification of this potentially regulatory monokine may permit a more accurate analysis of the role played by lactoferrin and other as yet unidentified factors in regulating steady-state granulopoiesis in vivo.

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