Granulocyte Macrophage Colony-Stimulating Activity Production by Cultured Human Thymic Nonlymphoid Cells Is Regulated by Endogenous Interleukin-1

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Supernatants of cultured human thymic nonlymphoid cells were assayed for granulopoietic factors using cultures of low density bone marrow mononuclear cells (LD-BMMC). Thymic nonlymphoid cell-conditioned medium (TNLC-CM) supported vigorous myeloid colony growth of LD-BMMC, and of LD-BMMC depleted of T lymphocytes and/or monocytes. Colony-stimulating activity (CSA) in TNLC-CM was abrogated by a highly specific neutralizing antisem to recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). TNLC-CM also enhanced colony growth in LD-BMMC stimulated by colony-stimulating activity from a giant cell tumor culture (GCT). The enhancing activity of TNLC-CM, unlike its CSA activity, required the presence of adherent cells in the marrow cell culture. The addition of anti-interleukin-1 (anti–IL-1) antibody to TNLC-CM inhibited the GCT-enhancing activity, but not the CSA. When the anti–IL-1 immunoglobulin was added directly to cultures of thymic nonlymphoid cells, GM-CSF production was completely inhibited, and the GCT-enhancing activity was neutralized. We conclude that an intercellular regulatory network exists in cultured thymic explants in which GM-CSF expression is induced by IL-1. In this system, the granulopoietic effect of IL-1 derives not from a direct effect on myeloid progenitors, but from its ability to recruit CSA production by other cells.

MATERIALS AND METHODS

Thymic nonlymphoid cell culture and collection of conditioned medium. Thymic nonlymphoid cell (TNLC) cultures were established according to previously described techniques.18 The monolayers represent outgrowths from explants of normal human thymus tissue obtained at the time of corrective cardiovascular surgery in otherwise normal children 3 years of age or less. After removal of the capsule and large blood vessels, the thymus tissue was minced into pieces about 1 to 3 mm in size, and approximately 30 to 40 pieces were placed in a 60 × 15-mm plastic petri dish, overlaid with serum-free RPMI 1640 (GIBCO, Grand Island, NY), and cultured at 37°C in a humid atmosphere of 5% CO2 in air. After 24 hours, RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Inglewood, CA) was used as the culture medium, with changes every five days. The outgrowing nonlymphoid cell monolayers were monitored by phase-contrast microscopy. By ten to 21 days the monolayers were usually 50% to 75% confluent, and the cultures as well as spent culture medium were free of detectable thymocytes.

The clonal growth of human myeloid cells in culture depends on the presence of a factor, or factors, termed colony-stimulating activity (CSA). There are at least four fully characterized proteins that exhibit CSA:1 granulocyte-macrophage colony-stimulating factor (GM-CSF),1,19 granulocyte colony-stimulating factor (G-CSF),4 interleukin-3 (IL-3), and colony stimulating factor-1 (CSF-1).5,6 Numerous sources of these and other hematopoietic factors have been identified.7 CSA has been found in culture media conditioned by a variety of tissues8 including stromal cells from bone marrow and spleen,9-11 mononuclear phagocytes,12,13 lymphocytes,14,15 fibroblasts,16 and vascular endothelial cells.17,18 Although the mechanisms regulating the production of these CSAs and the biologic importance of these cells as sources of CSA remain problematic,23 it is clear that production and release of CSA by some hematopoietic stromal cells are stimulated by the monokines interleukin-1 (IL-1) and tumor necrosis factor-alpha.20,21,22

The T lymphocyte likely plays an important role in granulopoiesis.13,17 In view of the importance of the thymus in the development of the T lymphocyte24,25 we reasoned that thymocyte-free cultures of thymic tissue might produce CSA. We describe here studies that support this hypothesis and demonstrate that the CSA produced in such cultures is largely GM-CSF, and that the production of this protein by thymic explant cultures is regulated by IL-1.

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human skin fibroblast monolayers (Human Genetic Mutant Cell Repository; repository #GM 5659) cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS.

TNLC-CM was exposed to murine monoclonal antibodies against IL-1α and IL-1β (kindly supplied by Dr Charles Dinarello, Tufts University) at final dilutions of 1:100 and 1:200, respectively, and incubated overnight at 4°C before study in the myeloid culture system. These antibodies have no direct inhibitory effect on colony formation supported by exogenous CSA.20

A single TNLC monolayer was grown in the presence of anti-IL-1, an immunoglobulin fraction from the serum of rabbits immunized with human monocytic-derived IL-1β (kindly provided by Dr Charles Dinarello). After 13 days of culture, the culture medium overlying the TNLC monolayer was changed in the usual way. The replacement medium was RPMI 1640 with 10% heat-inactivated FBS containing anti-IL-1 serum at a final dilution of 1:200. In a separate series of experiments this concentration of anti-IL-1 serum was shown to abrogate the CSA-inducing activity in supernatants of separate series of experiments this concentration of anti-IL-1 serum was shown to support colony formation by human marrow cells stimulated by CSA. Supernatant from this TNLC monolayer was collected six days later. Control CM were harvested from TNLC cultures of the same thymus grown without anti-IL-1, and from TNLC from the same thymus cultured after the 13th day in RPMI 1640 with 10% heat-inactivated FBS plus nonimmune rabbit serum at a final dilution of 1:200.

To determine the nature of the CSA present in TNLC-CM, a neutralizing polyclonal rabbit antibody against recombinant GM-CSF (kindly provided by Dr Bruce Attrill, AMGEN, Thousand Oaks, CA) was used. In a separate series of studies, overnight incubation with this antibody at a final dilution of 1:100 caused complete neutralization of granulopoietic GM-CSF activity in myeloid cultures performed with optimum concentrations of recombinant human GM-CSF. The antibody has no inhibitory effect on G-CSF or IL-3 (kindly provided by Genetics Institute, Boston).32

TNLC-CM was incubated with anti-GM-CSF antibody at a final dilution of 1:100 overnight at 4°C, before use in the myeloid colony assay.

Low density bone marrow mononuclear cells. Bone marrow from normal donors was aspirated into syringes containing sodium heparin (20 U/mL) after informed consent was obtained in accordance with the requirements of the human subjects research committee of the Oregon Health Sciences University. Aspirated marrow was diluted with an equal amount of phosphate buffered saline (PBS; pH – 7.2), and low density bone marrow mononuclear cells (LD-BMMC) (isotonic density ≤1.070 g/mL) were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Fine Chemicals, Upsalla, Sweden).33

Depletion of T Cells from LD-BMMC. Two methods of T cell depletion were used. The effectiveness of depletion and the effects of such depletions on colony growth were similar. In the first method, LD-BMMC were depleted of T cells by using a murine anti-T-cell monoclonal antibody (OKT3 [CD3]. Ortho Diagnostics, Raritan, NJ) plus rabbit complement. The concentrations of OKT3 and complement, which produced the maximum depletion of T cells, were determined in prior dose-response studies. Briefly, 1.5 × 106 LD-BMMC were mixed with OKT3 at a final dilution of 1:100 and held at 4°C for 60 minutes. The cells were then mixed with sterile baby rabbit serum as a source of complement (1:10 dilution; Pelfreez, Rogers, AR) and held for 60 minutes at 37°C in a humid environment, and then washed three times with PBS.

In the second method, LD-BMMC were depleted of T cells by sheep erythrocyte rosetting (SER). Briefly, LD-BMMC (106 cells/mL) were mixed with an equal volume of a 5% solution of sheep erythrocytes in RPMI 1640 supplemented with 15% FBS, pelleted by centrifugation of 200 g for eight minutes, incubated at 37°C in a humid environment for 30 minutes, then held at 4°C for 30 to 60 minutes. The mixture was gently resuspended and layered over Ficoll-Hypaque, centrifuged at 350 g for 25 minutes at room temperature, and the T cell depleted low density layer harvested.

Before depletion, LD-BMMC contained 45% to 71% T cells as determined by direct immunofluorescence staining with fluorescein-isothiocyanate (FITC) conjugated OKT3. The mean percentage depletion by OKT3 plus complement was 83% (range, 72% to 95%; n = 13) and by sheep erythrocyte rosetting was 71% (range, 55% to 82%; n = 5).

Depletion of monocytes by plastic adherence or monoclonal antibody. Two methods of monocyte depletion were used. First, LD-BMMC were depleted of monocytes by exposure to an anti-monocyte monoclonal antibody (OKM1, Ortho Diagnostics) plus complement as follows. LD-BMMC, 1.5 × 107, were mixed with OKM1 at a dilution of 1:10 and held at 4°C for one hour. Sterile rabbit complement was then added at a 1:3 dilution and the cells held at 37°C for an additional hour followed by three washes with PBS.

Before depletion, LD-BMMC contained 16% to 57% OKM1 positive cells as determined by FITC-OKM1 staining. The mean percentage depletion by monoclonal antibody plus complement was 56% (range, 11% to 98%; n = 6).

Second, LD-BMMC were depleted of monocytes by plastic adherence as follows. LD-BMMC (5 × 106 cells/mL) were layered onto plastic petri dishes previously coated with FBS, and incubated at 37°C in a humid environment for 60 minutes. The nonadherent monocyte-depleted cells were decanted and resuspended in RPMI 1640. The effectiveness of monocyte depletion by plastic adherence was evaluated by staining with butyrate esterase stain and by immunofluorescence staining with FITC-conjugated My-4 monoclonal antibody (Coulter, Inc). The mean percentage depletion was >80%.

Double-depleted LD-BMMC. Monocytes were removed from LD-BMMC by adherence as described above and the remaining cells were then depleted of T cells by the OKT3-complement lysis method.

Myeloid colony cultures. LD-BMMC or LD-BMMC depleted of T cells, mononuclear phagocytes, or both were cultured in soft agar in McCoy’s 5A medium34,35 enriched with amino acids, FBS (30%), and antibiotics (enriched medium, EM). Control CSA was from cultures of a giant cell tumor (GCT, GIBCO).36 EM was prepared by adding 2.2 g of NaHCO3 and 20 mL of Pen-Strep (10,000 U/mL of penicillin and 10,000 μg/mL of streptomycin) to 500 mL of McCoy’s 5A medium (all from GIBCO). To each 133 mL of this mixture were added 3.35 mL of sodium pyruvate (100 mmol/L), 2.7 mL of mixed essential amino acids (with L-glutamine), 1.35 mL of mixed nonessential amino acids (10 mmol/L), 1.35 mL of multivitamins, 1.35 mL of glutamine (29.2 mg/mL), and antibiotics (enriched medium, EM). Control CSA was prepared by the addition of 445 mg of dry agar (Difco, Detroit) to 60 mL of distilled water. Agar and EM were mixed in a 2:3 (vol/vol) ratio. Cells (106 cells in 0.1 mL) were added to 4.9 mL aliquots of combined agar and media. One milliliter of this cell suspension was cultured in 35 mm tissue culture plates. (In experiments to determine the optimum concentration of GCT, studies were performed using GCT in amounts from 0.02 mL to 0.3 mL. These studies demonstrated that the addition of 0.1 mL of GCT [9%, vol/vol] to cultures of LD-BMMC produced the maximal number of colonies under our culture conditions.) In control cultures, and in cultures examining the combined effect of TNLC-CM and GCT, cells were cultured with 0.1 mL of GCT (9%, vol/vol). Cultures were performed in duplicate or triplicate, and
incubated at 37°C in a humid environment of 7% CO2 in air. Colonies (aggregates of >20 cells) were enumerated on the seventh day of culture using a dissecting microscope, and results were expressed as the mean of duplicate cultures. Wright and butyrate esterase staining of sample colonies demonstrated both granulocyte and macrophage colonies.

In experiments examining the effect of TNLC-CM on myelopoiesis, four amounts of TNLC-CM or F-CM were studied with final concentrations of 17%, 23%, 28%, and 33%. A small number of cultures performed with higher concentrations of CM showed failure to gel.

Statistical analysis. Differences in colony growth were compared using the paired \( t \) test.

RESULTS

Colony stimulating activity in TNLC-CM. Ten experiments were performed comparing TNLC-CM and control F-CM to GCT as independent sources of CSA. As shown in Fig 1, TNLC-CM supported colony growth in cultures of both LD-BMMC and double-depleted LD-BMMC (DD-BMMC) equivalent to that produced by GCT alone. F-CM induced no appreciable colony growth.

Additive effects of TNLC-CM and GCT on LD-BMMC. The possibility that TNLC-CM could enhance myeloid colony formation in cultures containing an optimal amount of GCT was examined by adding varying amounts of TNLC-CM to cultures of LD-BMMC containing GCT. The number of colonies per \( 2 \times 10^3 \) LD-BMMC in cultures with both TNLC-CM and GCT was compared with the number of colonies in cultures with GCT alone, with the results expressed as the percent change from colony counts in cultures with GCT alone (Fig 2). TNLC-CM consistently enhanced GCT induced colony growth, but F-CM did not.

Additive effects of TNLC-CM and GCT on LD-BMMC depleted of T cells. Depletion of T lymphocytes from LD-BMMC by SER-density centrifugation or OKT3 plus complement lysis was compared in four experiments. Colony counts in GCT-supported cultures with various amounts of TNLC-CM and F-CM were similar for the two T-cell depletion methods (data not shown). In ten experiments, four concentrations of TNLC-CM and F-CM were added to LD-BMMC depleted of T cells. As shown in Fig 2, T-cell depletion of LD-BMMC did not abrogate the enhancing activity of TNLC-CM.

Loss of additive CSAs of TNLC-CM and GCT on LD-BMMC depleted of monocytes. The enhancing effects of TNLC-CM on GCT-stimulated cultures was completely abrogated by monocyte depletion. Cultures of LD-BMMC depleted of monocytes by OKM1 plus complement and stimulated by GCT alone produced a mean (±SD) of 145 ± 56 colonies. In GCT-supported cultures with four concentrations of TNLC-CM, the mean percentage change (±SD) in colony counts, compared with colony counts in cultures with only GCT, were 0% ± 10%, respectively. The differences between values for TNLC-CM and F-CM were significant (\( P < .001 \)) for the 28% and 33% concentrations. For T-depleted LD-BMMC, lines connect the means for each concentration of TNLC-CM (solid circles), or F-CM (open circles). Ten experiments were performed at each concentration. The differences between values for TNLC-CM and F-CM were significant (\( P < .02 \)) for the 23%, 28%, and 33% concentrations.

LD-BMMC depleted of T cells. As shown in Fig 2, T-cell depletion of LD-BMMC did not abrogate the enhancing activity of TNLC-CM.

Effect of antibodies against IL-1-α and IL-1-β on colony growth supported by TNLC-CM. TNLC-CM (28%) was
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Fig 3. LD-BMMC and DD-BMMC were cultured with TNLC-CM and with TNLC-CM treated with an overnight incubation with monoclonal antibodies against IL-1-α and IL-1-β (TNLC + anti-IL-1 [α and β]) to eliminate the effect of IL-1 in the TNLC on myeloid colony growth. The concentration of CM in each plate was 28%; experiments were performed in triplicate. The difference in colony counts for TNLC + TNLC-anti-IL-1 (α and β) in LD-BMMC was significant (P < .02).

Effect of anti-IL-1 antisera on release of CSA by TNLC monolayers and on the additive effect of TNLC-CM with GCT. In two experiments LD-BMMC were cultured individually with GCT and with 23% and 28% concentrations of TNLC-CM from TNLC monolayers exposed to a rabbit antisera against IL-1 (TNLC-anti-IL-1), or TNLC-CM from TNLC monolayers exposed to a control nonimmune rabbit serum (TNLC-CM-NRS). As shown in Fig 4, colony growth was seen in cultures of LD-BMMC stimulated by TNLC-CM and TNLC-CM-NRS, but not in cultures stimulated by TNLC-anti-IL-1. Results of an experiment using DD-BMMC as targets of CSA were the same (data not shown). As shown in Fig 5, preparation of TNLC-CM from thymic cultures grown in the presence of antibodies against IL-1 also abrogated that activity that enhanced colony growth in GCT-stimulated cultures.

Effect of antibody against GM-CSF on colony growth produced by TNLC-CM. LD-BMMC and DD-BMMC were cultured in two experiments with GCT alone, TNLC-CM (17% and 23% final concentrations), TNLC-CM after overnight incubation with antibody to GM-CSF (TNLC-anti-GM; 17% final concentration), and TNLC-CM after overnight exposure to control nonimmune rabbit serum (TNLC-CM-NRS; 17% final concentration). As shown in Fig 6, TNLC-anti-GM supported some colony growth in LD-BMMC but virtually none in DD-BMMC. This suggests that nearly all the CSA in TNLC-CM is GM-CSF, and further supports the hypothesis that some colony formation produced by TNLC-CM in LD-BMMC is due to CSA expressed by monocytes in the LD-BMMC under the influence of IL-1 present in the TNLC-CM. TNLC-CM-NRS supported colony growth in LD-BMMC and DD-BMMC similar to growth in cultures with TNLC-CM (data not shown).

Fig 4. LD-BMMC were cultured with medium conditioned by a thymic nonlymphoid cell monolayer exposed during culture to antibody against IL-1 (TNLC-anti-IL-1). Control cultures were grown with TNLC-CM, or medium conditioned by a thymic nonlymphoid cell monolayer exposed to nonimmune rabbit serum (TNLC-CM-NRS). Final concentrations of CM in marrow cultures were 23% and 28%. LD-BMMC were cultured with CSA from a giant cell tumor (GCT) for comparison. The mean of colony counts in paired cultures for each condition is shown.

Fig 5. Cultures of LD-BMMC were stimulated with CSA from a GCT. The enhancing effect of medium conditioned by thymic nonlymphoid cells (TNLC-CM) and medium conditioned by thymic nonlymphoid cells cultured in the presence of antibody to IL-1 (TNLC-anti-IL-1) was studied by adding TNLC-CM or TNLC-anti-IL-1 to GCT-stimulated cultures to a final concentration of 23%. Mean colony counts for paired cultures in each condition are shown.
DISCUSSION

We have confirmed recent reports that thymic nonlymphoid cells produce IL-1. In addition, TNLC-CM consistently supported GM colony growth in an assay in which few colonies formed spontaneously. F-CM contained no detectable CSA, confirming previous observations that products of auxiliary cells are required for the production of CSA by fibroblasts. Because TNLC-CM produced vigorous colony growth in cultures depleted of T lymphocytes and adherent cells, because IL-1 does not induce colony growth in such cultures and is known to induce GM-CSF expression, and because antibody directed against GM-CSF neutralizes colony growth stimulated by TNLC-CM, we conclude that TNLC-CM contains CSA and that most of the CSA is GM-CSF.

Synergy between hematopoietic growth factors has been reported previously. But it remains unclear whether the "synergy" of IL-1 and hematopoietic growth factors in stimulating granulocyte-monocyte colony growth reflects IL-1-induced alterations of progenitor responsiveness or IL-1-induced enhancement of growth factor production by auxiliary cells. Since IL-1 induces GM-CSF and G-CSF gene expression by a variety of cells, we tested the potential linkage of GM-CSF and IL-1 expression in the same culture system. A brief exposure to anti-IL-1 antibodies abrogated the additive effects of TNLC-CM and GCT, but did not alter the CSA activity of TNLC-CM; whereas incubation of thymic nonlymphoid cells with anti-IL-1 during five days of culture required to produce TNLC-CM eliminated the additive effects of TNLC-CM with GCT, and also resulted in the loss of CSA activity in the CM. Because we have shown that this anti-IL-1 antibody has no effect on the proliferation of human marrow cells in culture, we conclude that the production of GM-CSF by TNLC is IL-1 dependent. Because the IL-1 antibody abrogated the enhancing effect on colony growth stimulated by GCT, and because this additive effect was dependent on adherent cells, we propose that IL-1 is the enhancing factor in TNLC-CM and that it enhances colony growth by inducing CSA production by adherent cells. Specifically, cells present in the thymic nonlymphoid cell monolayer produce IL-1, which, in turn, induces the production of GM-CSF, and possibly other CSFs, by the same or different cells in the monolayer. Both IL-1 and GM-CSF are present in the TNLC-CM.

Lymphocytes are known to be a source of GM-CSF. This hematopoietic function of T lymphocytes may develop in precursor cells during passage through the thymus, much as the immunologic maturation of T cells is acquired during thymic residence. It is known that in a murine bone marrow transplant system the simultaneous administration of donor thymus tissue hastens the development of myelopoiesis. However, the respective roles of thymic epithelium or that of other thymic cells are difficult to define. Since the TNLC cultures that served as our source of CSA were free of detectable thymocytes, we propose that thymocytes may not be the only thymic component capable of CSA production. The production of GM-CSF by human thymic stromal cells has not been previously reported. A CSA capable of supporting colony growth of marrow cells was detected in cultures of murine thymic stroma, and macrophage CSF (CSF-1) has been detected in supernatants of thymocyte-free cultures of murine thymic stroma cells, and in the supernatants of subcultured human subcapsular thymic epithelial cells. IL-1 production has also been described in some thymic explant supernatants. Whether IL-1 production requires the presence of macrophages in the cultured thymic stroma is argued.

The cellular composition of our TNLC cultures is similar to primary cultures of human thymic stroma reported by other investigators, while it differs from murine TNLC cultures in the number and ratio of epithelial cells and monocyte/macrophages. We do not know which cellular component(s) in our TNLC monolayers is (are) responsible for the production of GM-CSF and IL-1. Brockband and vanPeery have reported that fibroblasts from various murine "hematopoietic organs" including thymus were capable of producing CSA. Since we used a skin-fibroblast CM as our control CM and found no myelopoietic effect and because fibroblasts are known not to produce IL-1, it is doubtful that the small number of fibroblasts in our thymic culture were responsible for IL-1 release. However, they could have responded to IL-1 (produced by other cells) by releasing GM-CSF. Although the cellular heterogeneity in our system prevents us from identifying the specific cell types involved in the IL-1 GM-CSF interaction we argue that our culture technique, because it is not reductionistic, reflects the in vivo state of thymic function.

Findings presented in Fig 3 are intriguing and suggest that colony growth inhibitors may be induced by TNLC-stimulated auxiliary marrow cells. The inhibitory activity may be IL-1-induced prostaglandin E2 but other possibilities exist. Three reports have suggested a suppressive role for thymic stromal cells on the production of CSA. Verma et al noted that preincubation of T lymphocytes with thymosin (fraction 5) resulted in a decrease in CSA production by a combined monocyte–T-lymphocyte culture system. This was similar to the effect of preincubation with concanavalin A and was attributed to a thymic activation of suppressor lymphocytes. Gruber reported that medium conditioned by murine TNLC inhibited colony growth of both granulocyte and monocyte-macrophage colonies in an assay system using known quantities of CSA. Tamir et al observed that the diffusible CSA produced by cultures of murine thymus containing epithelial cells, fibroblasts, and macrophages, was inhibited when the cocultured marrow cells were allowed close contact with thymic stromal cells. They proposed the presence of a low-titer inhibitor to explain this phenomenon. The mouse thymus cultures in Gruber's experiment contained only 20% epithelial cells; 80% of the cells were mononuclear phagocytes. The consistent difference in the number and ratio of epithelial cells and thymic monocytes between cultures of human and mouse TNLC may contribute to the differences in production of myelopoietic growth factors and inhibitors.

Although our TNLC cultures contain heterogeneous cell types, at least one of them is capable of producing IL-1 in
vitro and another (or perhaps the same cell type) is capable of responding to IL-1 by expressing the GM-CSF gene. Regardless of the nature of the active cell types in this thymic network, these findings may reflect important hematopoietic functions of the thymus in vivo. In view of evidence from studies on a human GM-CSF–dependent leukemia cell line, which suggests that GM-CSF may have an impact on the behavior of certain maturational stages of T lymphocytes, the expression by thymic nonlymphoid cells of the GM-CSF gene may even play a role in T cell maturation in the thymic microenvironment.

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