Regulation of Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor Expression by Oncostatin M

By T. Joseph Brown, Jingwen Liu, Carolyn Brashem-Stein, and Mohammed Shoyab

Oncostatin M (OM) is structurally and functionally related to a subclass of hematopoietic cytokines including leukemia-inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), granulocyte colony-stimulating factor (G-CSF), and interleukin-6 (IL-6). Using human endothelial cells (HEC) as a model for cytokine regulation of hematopoietic growth factor expression, we tested OM as an inducer of colony-stimulating activity. Colony-forming cell assays supplemented with culture supernatants from OM-treated HEC contained a threefold increase in colony-forming unit granulocyte-macrophage colonies. Specific immunoblot assay (enzyme-linked immunosorbent assay) of culture supernatants indicated that OM treatment of HEC resulted in a dose- and time-dependent increase in the accumulation of G-CSF and granulocyte-macrophage CSF (GM-CSF) (> 28-fold). The ED\textsubscript{50} for OM induction of G-CSF and GM-CSF protein expression was 17 and 7 pmol/L, respectively. Increased protein expression was associated with a similar increase in steady-state expression of G-CSF and GM-CSF mRNA. Furthermore, a period of 12 to 24 hours elapsed before there were measurable increases in CSF expression, suggesting that OM may stimulate CSF production through a mechanism requiring the synthesis or activation of a secondary mediator factor or pathway. These findings provide the first evidence that OM may regulate hematopoietic growth factors.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Cytokines and reagents. Endotoxin-free recombinant human OM was prepared as previously described.\textsuperscript{23} All other recombinant human cytokines and exon-specific cytokine oligonucleotides were purchased from R&D Systems (Minneapolis, MN).

Endothelial cell cultures. HEC were isolated from the umbilical vein as described.\textsuperscript{24} Cells were grown to confluence on gelatin-coated plasticware in CS-1-defined serum-free medium (Cell Systems). Cells received fresh unsupplemented media at least 12 hours before experiment.

Colony-forming cell assays. Marrow samples were obtained after informed consent and cells were separated by density gradient centrifugation.\textsuperscript{25} Bone marrow mononuclear cells (2.5 x 10\textsuperscript{6/mL}) were cultured in Iscove's modified Dulbecco's medium (IMDM), 0.9% methylcellulose (Terry Fox Cancer Center, Vancouver, BC, Canada) supplemented with 20% fetal bovine serum (FBS; HyClone, Logan, UT), 1% bovine serum albumin (BSA; InterGen Co, Purchase, NY), and 1 x 10\textsuperscript{-4} mol/L 2-mercaptopethanol (BioRad Laboratories, Richmond, CA) as previously described.\textsuperscript{26} Clarified supernatants of HEC-conditioned media (CM) were added as the only media supplement, unless otherwise specified. Cultures were incubated at 37°C in 5% CO\textsubscript{2} atmosphere. Observable macroscopic colonies were enumerated within 2 to 3 weeks.

From the Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA; and the Program in Pediatric Oncology, Fred Hutchinson Cancer Research Center, Seattle, WA.

Submitted November 30, 1992; accepted February 8, 1993.

Address reprint requests to T. Joseph Brown, Bristol-Myers Squibb RPI, 3005 First Ave, Seattle, WA 98121.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8201-00753.00/0

Blood, Vol 82, No 1 (July 1), 1993: pp 33-37

Blood, Vol 82, No 1 (July 1), 1993: pp 33-37
Immunosassays for CSFs. A sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of human G-CSF and GM-CSF in tissue culture media was performed on test samples according to the manufacturer's recommended procedure (R & D Systems). Briefly, 200-μL samples were incubated for 2 hours at 23°C in microtiter plates precoated with a specific monoclonal antibody (MoAb). After the washing procedure, microtiter wells were reincubated with specific horseradish peroxidase-linked polyclonal antibody. The plate was washed and then incubated for 20 minutes with a substrate solution containing hydrogen peroxide and tetramethylbenzidine. The enzymatic reaction was stopped with the addition of 2 N sulfuric acid. The color intensity of the reaction was determined at 450 nm using a spectrophotometric plate reader. Concentrations of both G-CSF and GM-CSF present in HEC-CM were determined by comparison to standard curves. Individual samples were run in duplicate. The minimal detectable concentrations of G-CSF and GM-CSF were 10 and 1.5 pg/mL, respectively.

Detection of mRNA for CSFs (Northern blot analysis). Cells were lysed in guanidium isothiocyanate and total RNA was isolated by ultracentrifugation through a cesium chloride cushion. Total RNA (20 μg/lane) was separated by electrophoresis in 1.2% agarose gels containing 6% formaldehyde and transferred onto nitrocellulose membranes. Gels were routinely stained with ethidium bromide before blotting to confirm that equivalent amounts of RNA were assayed. The prehybridization and hybridization was conducted in 50% formamide, 50 mmol/L sodium phosphate, pH 6.5, 5× SSC, 100 μg/mL of denatured salmon sperm DNA, and 6× Denhardt's solution overnight at 37°C. The filter was then washed with 6× SSC in 0.1% sodium dodecyl sulfate (SDS) for 20 minutes at 37°C. Subsequently, the filters were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) with an intensifying screen at −70°C for 1 to 3 days.

RESULTS AND DISCUSSION

Using cultured HEC as a model system for cytokine regulation of hematopoietic growth factor expression, we measured the ability of OM to induce colony-stimulating activity in colony-forming cell assays. HEC express receptors for OM but lack receptors for LIF, thus ensuring that OM action does not reflect cross-reactivity of OM with LIF receptors. Cultured HEC were treated with 100 ng/mL OM for 72 hours. The culture supernatants were collected and analyzed for the ability to stimulate isolated, human marrow progenitor cells to form colonies in semisolid media. Table 1 shows the increased number of granulocyte-macrophage colonies formed in the presence of OM-treated HEC-CM. Semisolid media supplemented with as little as 3% OM/HEC-CM contained three times as many macroscopic colonies as did the control 3% HEC-CM (16 ± 2 and 5 ± 1, respectively). There was a direct correlation between the amount of CM present and the development of granulocyte-macrophage colonies (colony-forming units granulocyte-macrophage [CFU-GM]). Because these assays were not supplemented with either IL-3 or erythropoietin, the increase in CFU-GM colonies was due to factors present in the OM-treated endothelial cell supernatants. In addition, OM was ineffective at directly stimulating colony formation in these assays, indicating that colony formation was not due to the presence of OM in the culture supernatants. Neither OM-stimulated HEC-CM nor nonstimulated HEC-CM supported erythroid colony formation (data not presented). All samples of HEC-CM were capable of supporting greater than 120 CFU-GM colonies in the presence of 100 ng/mL IL-3 (data not presented), possibly due to synergistic activity with constitutively expressed GM-
ONCOSTATIN M INDUCES CSF EXPRESSION

The level of colony formation in these experiments (31 ± 4 CFU-GM) was similar in magnitude to that reported by other investigators for a marrow response to G-CSF and GM-CSF in the absence of IL-3 and erythropoietin.30 The ability of OM to stimulate the production of specific hematopoietic growth factors in HEC was determined. OM induced the production level of both G-CSF and GM-CSF in a dose- and time-dependent manner. Cultured HEC were treated with increasing concentrations of OM. After 36 hours, the culture supernatants were collected and analyzed for G-CSF and GM-CSF content. As shown in Fig 1, OM treatment of HEC resulted in increased concentrations of both G-CSF and GM-CSF in the culture media as determined by immunoassay (ELISA). G-CSF concentrations in culture media increased from undetectable amounts to 11 ng/mL, whereas GM-CSF concentrations increased from 12 to 340 pg/mL (>28-fold increase) after OM treatment. The half-maximal effective dose (ED50) for OM-induced stimulation of G-CSF and GM-CSF levels was 0.5 ng/mL (16.7 pmol/L) and 0.2 ng/mL (6.7 pmol/L), respectively. Thus, the ED50 corresponded with occupancy of the high-affinity cell-surface receptor for OM.22 Maximal increases in hematopoietic growth factor levels occurred by treatment with 1 to 10 ng/mL (33 to 333 pmol/L) OM.

Fig 2. Time-dependent stimulation of (A) G-CSF and (B) GM-CSF production by OM. HEC were grown to confluence in 24-well tissue culture plates (1 × 10⁶ cells/mL/well). Culture supernatants were analyzed for G-CSF and GM-CSF content by ELISA technique at various times after treatment with (●) and without (○) 100 ng/mL OM. Each data point represents the mean value of triplicate samples ± SEM.

Culture supernatants were monitored by ELISA for G-CSF and GM-CSF content at various intervals after OM treatment (Fig 2). After 24 hours, both G-CSF and GM-CSF accumulated in the culture media at similar rates, although by 72 hours posttreatment, G-CSF concentrations had reached 20-fold higher levels than GM-CSF concentrations (50 ng/mL vs 2.5 ng/mL, respectively). It is interesting to note a significant lag period of 12 to 24 hours before detectable increases in G-CSF and GM-CSF content after OM treatment. By comparison, HEC were treated with 1 ng/mL of IL-1β, tumor necrosis factor-α (TNF-α), and OM for 18 hours, resulting in increases in G-CSF content of greater than 30-, 10-, and 4-fold, respectively (data not presented). These findings may indicate differences in the onset of responsiveness to various cytokines. The lag period after OM treatment is similar to that reported for TNF-α-induced CSF production in HEC, in which de novo protein synthesis was shown to be required.4 However, these kinetics are in contrast to those reported for IL-1–induced CSF expression in HEC4 and in long-term human marrow cultures,31 in which upregulation of CSF expression was detectable within 2 hours and maximal expression was detectable within 8 hours posttreatment. The delayed response to OM may indicate the involvement of an autocrine system in which synthesis of another cytokine is necessary to induce CSF gene expression. However, because OM did not induce IL-1α,
TNF-α, or LIF expression in HEC as determined by specific ELISA technique (data not presented), it is unlikely that these cytokines affect CSF expression in an autocrine manner.

To provide a molecular basis for the observed OM-induced increase in G-CSF and GM-CSF content of CM, we examined HEC for changes in expression levels of G-CSF and GM-CSF mRNA. HEC were treated with 100 ng/mL OM for various times. Cells were washed and solubilized and their total RNA was subjected to Northern hybridization analysis. As shown in Fig 3, radiolabeled oligonucleotides hybridized to specific mRNA species for G-CSF and GM-CSF. HEC constitutively expressed very low amounts of GM-CSF transcripts under these conditions, corresponding with the low constitutive levels of GM-CSF measured in culture supernatants. The apparent induction of CSF transcripts by OM occurred between 8 and 24 hours posttreatment. These findings are consistent with the lag period observed in CSF protein expression and indicate differential mechanisms of action between OM and IL-1 at inducing CSF expression in HEC.

The cytokine network is thought to play an important role in regulating hematopoiesis. Similar to IL-1 and TNF, OM is expressed in macrophages and T lymphocytes in response to activating stimuli and may therefore be available to act on bone marrow stroma during inflammatory/immune responses and hemostatic imbalance. The structural and functional similarity of OM and its receptor complex with that of IL-6 and LIF suggests a direct role for OM within the bone marrow as a maturation factor for megakaryocytes.23-30 The findings reported here suggest an indirect role for OM as an activator of mesenchymal phenotypes involved in maintaining the hematopoietic microenvironment.

REFERENCES

25. Lithow MR, Brashem-Stein C, Andrews RG, Bernstein ID:
Proliferative responses to IL-3 and G-CSF distinguish a minor subpopulation of CD34 positive marrow progenitors that do not express CD33 and a novel antigen. Blood 77:2354, 1991


Regulation of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor expression by oncostatin M

TJ Brown, J Liu, C Brashem-Stein and M Shoyab