Cloning and Expression of Murine Interleukin-1 Receptor Antagonist in Macrophages Stimulated by Colony-Stimulating Factor 1

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Colony-stimulating factor 1 (CSF-1) can act on mature macrophages to modulate their production of inflammatory cytokines. A cDNA encoding the interleukin-1 receptor antagonist (IL-1Ra) was cloned by subtractive hybridization from a CSF-1–stimulated murine macrophage cell line, sequenced, and expressed in mammalian and bacterial cells. Mouse IL-1Ra is a 22-Kd glycoprotein that is 76% identical to its human counterpart, shows considerably less similarity to IL-1α and IL-1β, and competes with IL-1α for binding to the type 1 IL-1 receptor normally expressed on T cells and fibroblasts. CSF-1 treatment of mouse bone marrow–derived macrophages led to a rapid and sustained increase in IL-1Ra mRNA during the G1 phase of the cell cycle as well as to increases in mRNAs encoding IL-1α and IL-1β. Cycloheximide inhibited CSF-1–induced IL-1α mRNA synthesis, but augmented IL-1β mRNA production and did not affect induction of IL-1Ra mRNA. No IL-1Ra mRNA was observed in CSF-1–stimulated mouse fibroblasts engineered to express CSF-1 receptors, demonstrating that its regulation depends on cell context and can be dissociated from the proliferative response. In agreement, bacterial lipopolysaccharide, a nonmitogenic activator, also induced IL-1Ra and IL-1 mRNAs in macrophages. Unlike IL-1α and β, IL-1Ra contains a signal peptide. The kinetics of its induction and its ability to gain access to the secretory compartment imply that IL-1Ra may be secreted more efficiently than IL-1, and suggest that macrophages both positively and negatively regulate the IL-1 response.

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THE HEMATOPOIETIC growth factor, colony-stimulating factor 1 (CSF-1), induces the formation of macrophages from bone marrow precursor cells and is required for the continued survival of mature circulating monocytes and tissue macrophages. CSF-1 is a 90-Kd homodimeric glycoprotein produced primarily by fibroblasts and endothelial cells, which exerts its effects by binding to a single class of high-affinity receptors expressed on cells of the mononuclear phagocyte lineage. Apart from its ability to support the proliferation, differentiation, and viability of mononuclear phagocytes during hematopoiesis, CSF-1 induces the production of other monokines, including interleukin-1 (IL-1); granulocyte CSF (G-CSF); interferon-γ; and tumor necrosis factor-α (TNF-α).

The macrophage CSF-1 receptor (CSF-1R), encoded by the c-fms proto-oncogene, exerts its effects through its intrinsic, ligand-activated tyrosine kinase, which triggers a series of as yet incompletely characterized biochemical reactions that ultimately lead to the induction of CSF-1–responsive genes. The pleiotropy of the receptor-mediated response is mediated, at least in part, by the ability of the activated CSF-1R kinase to associate with and phosphorylate different cellular proteins whose combinatorial actions determine the overall nature of the biologic response. After expression of c-fms genes in naive, receptor-negative cells, CSF-1 can induce either proliferation or differentiation, depending on the nature of the target cell.

Certain of the genes regulated by CSF-1, such as c-fos, c-junB, and c-myc, are activated in many cell types as part of their program of immediate early responses to growth factor stimulation. In contrast, a different subset of CSF-1–responsive genes would be expected to participate in specific macrophage responses. By using a cDNA library prepared from a CSF-1–stimulated murine macrophage cell line, we have begun to isolate and characterize inducible genes whose activities mediate macrophage effector functions. We show that one such gene encodes the IL-1 receptor antagonist (IL-1Ra), a secreted protein that competes with IL-1 for binding to its receptor. The fact that both IL-1Ra and IL-1 genes are coinduced in macrophages is paradoxical, given their opposing biologic activities on diverse target cells.

MATERIALS AND METHODS

Cells and culture conditions. SV40-immortalized murine BAC1.2FS macrophages depend on CSF-1 for their proliferation and survival in culture. They arrest in the early G1 phase of the cell cycle when deprived of CSF-1 and die within 36 hours in the absence of the growth factor. Like normal murine bone marrow–derived macrophages, G1-arrested BAC1.2FS cells restimulated with mitogenic doses of CSF-1 progress synchronously into S phase 10 to 12 hours later, after which they can complete cell division in the absence of the growth factor. BAC1.2FS cells were grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, and 25% L-cell-conditioned medium as a source of murine CSF-1. Mouse NIH-3T3 fibroblasts and C127 epithelial cells were maintained in the same medium without CSF-1 supplements. Chinese hamster ovary (CHO) cells were grown in Ham’s F12 medium supplemented with 10% FCS and 20 mmol/L HEPES buffer (Flow Laboratories, McLean, VA).

Molecular cloning of murine IL-1Ra cDNA from CSF-1–stimulated macrophages. Polyadenylated RNA was extracted from BAC1.2FS cells that had been starved for CSF-1 for 18 hours and then

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restimulated with the growth factor for 3 hours. A cDNA library created in bacteriophage λgt10 and containing approximately 1 x 10^6 clones was screened with a subtracted radiolabeled probe enriched for sequences expressed in the induced cells. The probe was prepared in a reaction mix containing [32P]dCTP and actinomycin D by reverse transcription of polyadenylated RNA from purified and assayed for their ability to cross-hybridize to one soluble competitor. Column fractions, serially diluted in 25 mmol/L HEPES buffer, pH 7.2, containing 1% bovine serum albumin, were incubated for 1 hour at 22°C with purified [%1-labeled IL-1α (2,000 cpm) and assayed for their ability to compete with radiolabeled IL-1α for binding assays. Conditioned medium from cell lines engineered to produce IL-1α was concentrated 50-fold by pressure dialysis and tested for its ability to compete with radiolabeled IL-1α in binding to its receptor. Media from untransfected cells were similarly concentrated and used as controls. CHO cells engineered to express a cloned mouse type I IL-1 receptor and expressing approximately 1,400 IL-1 binding sites per cell were incubated for 1 hour at 22°C with purified [%1-labeled IL-1α (2,000 Ci/mmol; Amersham, Arlington Heights, IL), either in the absence or presence of unlabeled competitors. The cells were washed, lysed, and the amount of bound radioactivity was determined. In some experiments, aliquots of IL-1α-conditioned medium were fractionated by high-performance liquid chromatography (HPLC) on a TSK G3000SW column (Toso, Japan) using internal protein standards of known molecular weight to estimate the mass of the soluble competitor. Column fractions, serially diluted in 25 mmol/L HEPES buffer, pH 7.2, containing 1% bovine serum albumin, were assayed for their ability to compete with [%1-labeled IL-1α for receptor binding as described above.

**Production of antiserum to murine IL-1α.** An EcoRI fragment containing the complete open reading frame of mouse IL-1α (nucleotides 80 to 644, Fig 1A) was treated with the Klenow fragment of DNA polymerase I, ligated to BamHI linkers, and cloned into the unique BamHI site of a feline sarcoma virus expression plasmid. The DNA was cotransfected into NIH-3T3, C127, and CHO cells, together with a second plasmid (pSV2neo) containing the neomycin-resistance (neo) gene. Transfected cells were selected for 3 weeks in medium containing 800 μg/mL G418 (Geneticin; GIBCO, Grand Island, NY), and resistant cells were pooled and confirmed by Northern blotting analysis to produce mouse IL-1α mRNA.

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intramurally injected with 10 μg of purified protein emulsified in complete Freund’s adjuvant and were boosted at 3-week intervals with 2 μg protein in incomplete adjuvant. The animals were bled 1 week after each boost, and antisera were tested for their ability to immunoprecipitate metabolically labeled IL-1Ra.

Metabolic labeling. Cells were metabolically labeled for various times with 150 μCi/mL each of [35S]cysteine (600 Ci/mmol) and [35S]methionine (800 Ci/mmol) (Amersham), lysed with detergent, and the lysates immunoprecipitated with rabbit antisera to IL-1Ra or preimmune serum as described.19 Radiolabeled proteins were separated on denaturing 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS), and the proteins were detected by autoradiography of the dried slab gels.77

RESULTS

Polyadenylated RNA, isolated from murine BAC1.2F5 macrophages that had been growth-arrested in early G1 by CSF-1 deprivation and then restimulated with the growth factor for 3 hours, was used as a template to construct a λgt10 bacteriophage library. Five families of clones were derived using a subtracted cDNA probe enriched for sequences expressed in induced cells, one of which encoded murine IL-1Ra (see below). The sequence of this clone (Fig 1A) is 1,951 nucleotides in length and includes a single long open reading frame beginning with an ATG codon at residues 98 through 100 and terminating with a TAG codon at nucleotides 632 through 634. The putative 5’-untranslated sequence of 97 nucleotides includes two additional ATG codons in an alternative reading frame (nucleotides 18 through 20 and 57 through 59) followed by a TAA stop codon at nucleotides 129 through 131. None of the three ATG codons exactly conform to consensus sequences for translation initiation (PuCCATGG), although the second and third ATGs are more closely matched than the first. Therefore, initiation at the two upstream sites might decrease the rate of translational initiation at the third ATG. The open reading frame is followed by an untranslated sequence of 1,320 nucleotides, which included a signal for polyadenylation (residues 1921 through 1926) followed by 3’ terminal poly(A) sequences. AUUUA-rich motifs found in the 3’-untranslated segments of many other cytokine mRNAs, and that are presumed to be important in targeting them for degradation,39 were not identified within the 3’-untranslated region.

Comparison of the nucleotide sequence shown in Fig 1A with others in the GenBank Database (release 63; Los Alamos National Laboratory, Los Alamos, NM) showed homology to those of human IL-1α and β. This prompted us to compare the sequence with that of human IL-1Ra, which had been recently cloned from monocytes and, independently, from U937 leukemia cells.23 The predicted 178-amino acid sequence encoded by the murine cDNA was found to be 76% identical to human IL-1Ra (Fig 1B). In contrast, the predicted sequence of human IL-1Ra shows only 26% identity to human IL-1β and 19% to human IL-1α, which are identical to each other at only 26% of their residues.40,41 Both the human and murine IL-1Ra clones are predicted to contain hydrophobic signal sequences at their aminotermini, but the mouse precursor contains 26 rather than 25 N-terminal hydrophobic residues and is therefore one amino acid longer than its human counterpart (Fig 1B). Cleavage of the human polypeptide by leader peptidase between residues 25 and 26 (arrow, Fig 1B) results in a mature 152-amino acid polypeptide of 17.1 Kd with an N-terminal sequence corresponding to that of the purified monocyte-derived receptor antagonist.2,23 The mouse and human IL-1Ra polypeptides each contain a single canonical site for addition of an asparagine-linked oligosaccharide chain (star, Fig 1B). Glycosylation of the human polypeptide results in the production of a mature extracellular protein of 22 Kd.20,21

To demonstrate that the murine cDNA encoded a biologically active IL-1 antagonist, we cloned it into a mammalian expression vector and introduced the gene into mouse C127 epithelial cells and CHO cells. Because the 5’-cloning site used to insert the IL-1Ra cDNA into the retroviral vector corresponded to nucleotide 80 (Fig 1), neither of the two upstream ATGs was included. When used to compete for the binding of radiolabeled human IL-1α to the cloned murine IL-1 receptor expressed on CHO cells, specific inhibition of IL-1 binding was observed using conditioned medium from vector-infected, but not control, cultures (Fig 2A). Partial purification of the competing activity by gel filtration indicated that the protein in the active fractions had an apparent molecular weight of about 20 Kd (Fig 2B), consistent with the predicted mass of the monomeric, extracellular glycoprotein. We also expressed the IL-1Ra cDNA as a bacterial fusion protein and prepared antiserum to the engineered polypeptide. Metabolic labeling of mammalian cells engineered to produce IL-1Ra followed by immunoprecipitation of cell lysates using this antiserum confirmed the presence of a specifically precipitable polypeptide of 22 Kd (Fig 2C). However, the undenatured bacterial fusion protein was not biologically active in competing with IL-1α for binding to its receptor on mammalian cells. It is unlikely that this was due to its lack of carbohydrate, because enzymatically deglycosylated human IL-1Ra retains biologic activity,25 so we suspect that the glutathione S-transferase moiety inhibits the binding.

To study the kinetics of induction of IL-1Ra in macrophages, starved BAC1.2F5 cells were stimulated with CSF-1 for various times, and their RNA was isolated and subjected to Northern blotting analysis using the radiolabeled murine IL-1Ra cDNA as a probe. Figure 3A shows that IL-1Ra transcripts of about 2.1 kb were detected within 10 minutes of CSF-1 stimulation, and their level continued to increase for 5 hours. By contrast, expression of c-fms (encoding CSF-1R) remained relatively constant throughout the same period. BAC1.2F5 cells stimulated for 3 hours with CSF-1 in the presence of cycloheximide showed a similar elevation in their level of IL-1Ra transcripts (Fig 3A). The fact that new protein synthesis was not required shows that induction of IL-1Ra is part of the immediate early response to CSF-1 in these cells.

The kinetics of induction of IL-1Ra were also studied in normal bone marrow-derived macrophages. Cells harvested from the long bones of Balb/c mice were propagated for 10 days in serum-containing medium containing CSF-1 as the only exogenous hematopoietin. Under these conditions,
Fig 2. Characterization of the mouse IL-1Ra protein. (A) Competition of IL-1Ra and \(^{125}\text{I}\)-IL-1\(\alpha\) for receptor binding. CHO cells expressing the mouse type I IL-1 receptor were incubated with \(^{125}\text{I}\)-IL-1\(\alpha\) (approximately 1 ng) in the presence of purified IL-1 (○) at the concentrations shown. Conditioned media from control CHO cells (△) or CHO cells engineered to produce murine IL-1Ra (●) were concentrated 50-fold and tested at different dilutions for competing activity. Log 1 indicates competition obtained with the undiluted 50X concentrate that was diluted through a 10-fold range (to log 0). (B) Conditioned medium from control CHO cells (○) or from those engineered to produce murine IL-1Ra activity (●) was fractionated by molecular sieving on an HPLC column, and fractions were tested at equal dilution for competition with \(^{125}\text{I}\)-IL-1\(\alpha\) for receptor binding. The peak competing activity was obtained for proteins of approximately 20 Kd. (C) Control C127 cells (lanes 1 through 3) or those engineered to express murine IL-1Ra (lanes 4 through 6) were metabolically labeled for 30 minutes (lanes 1, 2, 4, and 5) or 2 hours (lanes 3 and 6), lysed, and immunoprecipitated with either preimmune (lanes 1 and 4) or immune (lanes 2, 3, 5, and 6) serum. Radiolabeled proteins were separated on denaturing polyacrylamide gels containing SDS and detected by autoradiography (4-day exposure) of the dried slab gel. The position of specifically precipitated IL-1Ra is indicated by the arrow at the right margin; the positions of proteins of known molecular weight are shown at the left.

pure populations of mononuclear phagocytes are obtained. As expected, these cells expressed typical markers of the mononuclear phagocyte lineage, including CSF-1R (Fig 3B). When starved of CSF-1 for 18 hours and restimulated for various times, IL-1Ra transcripts were induced within 15 minutes, reached a maximum at 10 hours (approximating the G1/S transition), and declined. The initial rapid phase of induction could involve mRNA stabilization, but transcriptional regulation would logically contribute to the relatively long duration of the response. However, we have not analyzed the process using nuclear run-on experiments and therefore cannot gauge the relative contributions of transcriptional and posttranscriptional mechanisms to IL-1Ra mRNA induction.

IL-1 mRNAs were also induced by CSF-1, with peak levels of IL-1\(\alpha\) (Fig 3B) and IL-1\(\beta\) (not shown) mRNAs detected 6 hours after stimulation. Bacterial lipopolysaccharide (LPS) treatment of CSF-1-starved cells also augmented the levels of IL-1Ra and IL-1 mRNAs, but in this case the relative amounts of the IL-1 mRNAs exceeded that of IL-1Ra (Fig 3B). Induction of IL-1a mRNA was inhibited by cycloheximide, whereas the levels of IL-1\(\beta\) mRNA were further increased by treatment of cells with the protein synthesis inhibitor (data not shown). Thus, induction of the IL-1\(\beta\) mRNA, like IL-1Ra, occurs as part of the immediate early response to CSF-1, whereas induction of IL-1a requires new protein synthesis. The different patterns of IL-1a, IL-1\(\beta\), and IL-1Ra mRNA expression in response to cycloheximide treatment indicate that their rates of mRNA turnover are independently regulated.

DISCUSSION

IL-1 affects a broad range of local and systemic hematologic, immunologic, neurologic, and metabolic activities. During the inflammatory response, the two structurally distinct forms of IL-1, IL-1\(\alpha\) and IL-1\(\beta\), are synthesized by mononuclear phagocytes in response to endotoxin and TNF-\(\alpha\), and despite their limited sequence homology to one another, they act on the same target cells by binding to common receptors. The spectrum of biologic responses to IL-1\(\alpha\) and \(\beta\) depends on the nature of the responding cells. For example, both IL-1 species induce the production of IL-2 and upregulate expression of the IL-2 receptor in T cells. They each stimulate the antifibrinolytic and adhesive activities of endothelium and augment the synthesis of prostaglandins and proteases by fibroblasts and chondrocytes. The activities of IL-1 on T cells and fibroblasts are
mediated by the type I IL-1 receptor, but a second class of IL-1 receptors (type II) is expressed on both B lymphocytes and macrophages.

The apparent complexity of the cytokine networks that regulate IL-1 production by macrophages has been compounded by the recent identification of the human IL-1 receptor antagonist. IL-1Ra synthesis is rapidly induced in human monocytes or in U937 leukemia cells stimulated by aggregated IgG, granulocyte-macrophage CSF (GM-CSF), LPS, or phorbol esters. The secreted 22-Kd glycoprotein binds to type I IL-1 receptors with the same affinity as either IL-la or p. Although human IL-1Ra does not bind to murine B cells or neutrophils, it can compete with IL-1 for binding to human type II IL-1 receptors. Therefore, we assume that murine IL-1Ra can bind in a similar species-specific manner to IL-1 receptors expressed on mouse B cells and macrophages.

Induction of IL-1Ra mRNA is part of the immediate early response of quiescent mouse macrophages brought into cycle by CSF-1. CSF-1 induced a rapid and prolonged increase in IL-1Ra mRNA levels that peaked 10 hours later during the late G1 phase of the cell cycle. Murine IL-1Ra is 76% identical to its human counterpart but shows considerably less sequence homology to either IL-1α or β, and, as predicted, was able to compete with radiolabeled IL-1α for binding to CHO cells engineered to express type I mouse IL-1 receptors. Molecular sieving analysis showed that the soluble IL-1 competing activity produced by mammalian cells expressing murine IL-1Ra cDNA was similar in molecular weight to the protein engineered in bacteria, indicating that murine IL-1Ra is biologically active in its monomeric 22-Kd form. Although the CSF-1-induced production of IL-1Ra was associated with the release of macrophages from growth arrest, IL-1Ra mRNA was also induced by LPS, a macrophage activator that inhibits CSF-1-induced mitogenesis by triggering the proteolytic cleavage of CSF-1R from the cell surface. Conversely, whereas transduction of the human c-fms gene into mouse NIH-3T3 fibroblasts enables them to proliferate in response to human recombinant CSF-1, treatment of such cells with CSF-1 did not induce IL-1Ra mRNA (negative data not shown) under conditions where c-fos and junB are rapidly induced. Thus, the regulation of IL-1Ra by CSF-1 depends on cell context and appears to reflect a macro-

Fig 3. Expression of IL-1Ra and IL-1 mRNAs in mouse macrophages. (A) Northern blot analysis was performed with the indicated probes using RNA from BAC1.2F5 mouse macrophages. Quiescent cells were stimulated for the indicated times (minutes) with CSF-1 in the absence (left) or presence (right) of cycloheximide (25 μg/mL). The mRNA band corresponding to CSF-1R (c-fms) was estimated to be approximately 4 kb. Autoradiographs were exposed for 18 hours. (B) Normal mouse bone marrow-derived macrophages, starved of CSF-1 for 18 hours, were restimulated with CSF-1 or LPS (10 μg/mL) for the indicated times in minutes or hours (h). The same blots were probed as indicated at the left with radiolabeled IL-1Ra, IL-1α (exposure times 18 hours), or c-fms cDNAs (exposure time 3 days).
phage effector function rather than the proliferative re-
ponse to the growth factor.

IL-1Ra contains an aminoterminal signal peptide and
must be transported through the secretory pathway because
it undergoes addition of a single asparagine-linked oligosac-
charide chain.\(^{21}\) Despite their numerous systemic activities,
however, both the \(\alpha\) and \(\beta\) forms of IL-1 lack signal
sequences and are synthesized from larger 31- to 34-Kd
precursors whose intracellular transport and proteolytic
processing to mature 17-Kd secreted forms are determined
by as yet unexplained mechanisms. IL-1 precursors do not
gain access to the endoplasmic reticulum.\(^{31,34}\) Indeed, fibro-
blasts stably expressing cloned IL-1\(\alpha\) or \(\beta\) cDNAs fail to
secrete processed 17-Kd IL-1 into the culture medium,
although their precursors accumulate in the cytoplasm.\(^{31,35}\)
Even in activated human monocytes, the half-life for
secretion of IL-1 is comparatively slow, measured at about 3
hours for IL-1\(\beta\) and considerably longer for IL-1\(\alpha\).\(^{35}\) Several
lines of evidence suggest that IL-1\(\alpha\) can be expressed as a
bioactive membrane-bound form at the cell surface, which
is accessible to cleavage by extracellular proteases and
blocked in its activity by appropriate antibodies,\(^{46,59}\) but
mechanisms governing membrane association have not
been defined. Unglycosylated IL-1 precursors may be trans-
ported to the cell surface through an alternative vesicular
pathway and cleaved just before release.\(^{57}\) Although process-
ing and release are not obligatory steps, they are triggered
by treatment of cells with calcium ionophores, heat shock,
and inhibitors of either protein synthesis or oxidative
phosphorylation. Moreover, drugs such as blefeldin A and
monensin, which block protein transport within the classi-
cal secretory pathway and inhibit the production of cyto-
kines such as IL-6 and TNF-\(\alpha\), accelerate the proteolytic
processing and release of IL-1B.\(^{57}\)

CSF-1 induction of IL-1Ra and IL-1B mRNA in mouse
macrophages is not inhibited by cycloheximide, whereas
production of IL-1\(\alpha\) is contingent on new protein synthesis.
Although both the IL-1Ra and IL-1\(\beta\) genes participate in
the immediate early response to CSF-1, monocytic cell lines
such as U-937 that secrete IL-1Ra after induction,\(^{39}\) synthe-
size but neither process nor secrete IL-1\(\beta\).\(^{57}\) Taken

NOTE ADDED IN PROOF

The nucleotide sequence of murine IL-2Ra has been filed in
GenBank (Los Alamos National Laboratory, Los Alamos, NM)
under accession number M64404.

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