Molecular Physiology of Granulocyte-Macrophage Colony-Stimulating Factor

By Judith C. Gasson

HEMATOPOIESIS IS A quantitatively imposing process in the adult, where about $3 \times 10^8$ erythroid cells/kg and $10^9$ myeloid cells/kg are generated each day. Twenty-five years ago, in vitro assays were developed to detect factors that could stimulate the growth of colonies from immature bone marrow (BM)-derived progenitors. These systems have made it possible to identify and characterize the factors regulating myelopoiesis. The application of molecular biologic approaches to study hematopoiesis facilitated the cloning of genes encoding the hematopoietic growth factors and their production in mammalian cells, yeast, and bacteria in quantities sufficient for in vitro and in vivo studies. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of a family of glycoprotein cytokines that have potent effects in stimulating proliferation, maturation, and function of hematopoietic cells. Although GM-CSF was the first, several hematopoietic growth factors are now used in clinical trials. Most of the effects observed using GM-CSF in vitro have been shown to occur in vivo either in animal models or in human subjects. In this review, I shall summarize the basic science studies on GM-CSF, because others have recently reviewed the literature on the clinical applications of GM-CSF and other hematopoietins.

GENOMIC ORGANIZATION AND CHROMOSOMAL LOCALIZATION

Murine and human GM-CSFs (hGM-CSFs) are encoded by genes that are highly homologous in both the coding and noncoding regions. Both genes are approximately 2.5 kilobase pairs (kbp) in length, consisting of four exons separated by three intervening sequences. In the mouse, the GM-CSF gene has been localized to chromosome 11, and linkage between murine GM-CSF and interleukin-3 (IL-3 or multi-CSF) has been demonstrated. The hGM-CSF gene has been mapped to the long arm of chromosome 5 (5q21-2q32). This localization is interesting because it is 9 kbp downstream from the hIL-3 gene. hIL-4, IL-5, macrophage CSF (M-CSF), c-fms (the M-CSF receptor), and an early growth response gene (EGR-1) are also localized to the long arm of chromosome 5. Interstitial deletions in this region are seen predominantly in therapy-related myelodysplastic syndromes and acute leukemias, as well as in the 5q syndrome. Detection of a portion of a chromosome bearing genes encoding hematopoietic growth factors as well as receptors is intriguing in light of the hematologic abnormalities associated with 5q-syndrome (a refractory anemia and morphologic abnormalities of their megakaryocytes). It is possible that deletion of this region allows expression of aberrant or recessive alleles that are involved in the generation of abnormal hematopoiesis.

GM-CSF EXPRESSION

Over 15 years ago, Cline and Golde demonstrated that human lymphocytes produce colony-stimulating activity upon stimulation with phytohemagglutinin (PHA). GM-CSF can be synthesized by a variety of cell types in response to specific activating signals. T cells, macrophages, mast cells, endothelial cells, and fibroblasts can be induced to accumulate GM-CSF mRNA and secrete GM-CSF protein (see Table 1). T cells and macrophages are directly activated by immune or inflammatory stimuli. GM-CSF production by endothelial cells and fibroblasts appears to be induced by monokines such as IL-1 and tumor necrosis factor (TNF). If one considers the potential physiologic role of GM-CSF in enhancing host defense, production of this factor by cells that are sensitive to immune challenge is certainly teleologically consistent. GM-CSF does not appear in the circulation at detectable levels. Therefore, unlike a classical endocrine hormone, GM-CSF behaves according to a paracrine model where the substance is produced and acts locally.

Expression of GM-CSF protein may also play a role in pathologic processes (Table 1). Autocrine expression of GM-CSF in myeloid leukemia cells and cell lines has been proposed to play a role in neoplasia (discussed below); expression of GM-CSF has also been documented in...
induced murine monocyte tumors may be the result of aberrant regulation of GM-CSF mRNA stability.25

Transcriptional regulation of GM-CSF expression plays an important role in activated T lymphocytes. A wide variety of approaches and cell types have been used to identify the critical regions of the GM-CSF gene controlling its expression. Sequence analyses comparing upstream regions of murine and hGM-CSF genes with each other and other lymphokine genes showed significant homologies in the 330 bp upstream of the putative TATA box (see Fig 1).27 This includes a GC-rich region and a decanucleotide consensus sequence described by Stanley et al that can be found in the murine and hGM-CSF, IL-3, and IL-2 gene promoters.7 Based on the apparent homologies of the murine and hGM-CSF promoter regions, several groups have used mobility shift assays to look for binding proteins that may regulate expression of these genes.

In addition to the conserved decanucleotide consensus sequence, cytokine consensus 1 (CK-1),26 also referred to as conserved lymphokine element 1 (CLE-1),27 which is conserved in murine and hGM-CSF, IL-3, IL-2, and granulocyte CSF (G-CSF), a second region of homology, CK-2 or CLE-2, is conserved in murine and hGM-CSF, IL-3, IL-2, and granulocyte CSF (G-CSF) and mouse GM-CSF.28 Recently, NF-GMa activity was shown to be increased by TNF treatment of embryonic fibroblasts. Thus, Shannon et al proposes that NF-GMa is a novel transcription factor, inducible by TNF treatment, that binds to CK-1 and is important in regulation of hematopoietic growth factor genes in fibroblasts.28

James and Kazenwadel performed mobility shift and methylation interference assays using a murine GM-CSF promoter fragment.29 With nuclear extracts from a lectin-stimulated murine T-cell line, they observed protein binding in the GC-rich region just downstream of the CK-2 sequence described above (see below and Fig 1). This region has also been implicated in inducible GM-CSF transcription in a human T-cell line by Miyatake et al, who used recombinant constructs containing murine GM-CSF promoter sequences.20,28 Recombinant constructs were transiently transfected into the human T-cell line, Jurkat. Cells were stimulated with phorbol esters and ionophore to mimic T-cell activation, and the ability of recombinant constructs to drive expression of the bacterial CAT gene was determined. The authors found that CLE-2 (or CK-2) was important for inducible expression of recombinant constructs containing the murine GM-CSF promoter in the

## Table 1. Cells Expressing GM-CSF

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Stimuli</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>T lymphocytes</td>
<td>antigen, lectin, CD28, IL-1, HTLV</td>
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<tr>
<td>B lymphocytes</td>
<td>LPS, TPA</td>
<td>165</td>
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<tr>
<td>Macrophages</td>
<td>LPS, FCS, phagocytosis, adherence</td>
<td>20,166,167</td>
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<tr>
<td>Mast cells</td>
<td>IgE, calcium ionophore</td>
<td>168,169</td>
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<tr>
<td>Fibroblasts</td>
<td>TNF, IL-1, TPA</td>
<td>170-174</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>TNF, IL-1, TPA, modified LDL</td>
<td>170,175-180</td>
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<tr>
<td>Mesothelial cells</td>
<td>EGF + TNF</td>
<td>181</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>PTH, LPS</td>
<td>182</td>
</tr>
<tr>
<td>Potential Pathophysiologic Sources of GM-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>TNF, adherence, IL-1</td>
<td>50-57,183,184</td>
</tr>
<tr>
<td>Rheumatoid synovium</td>
<td></td>
<td>118,119</td>
</tr>
<tr>
<td>Solid tumors</td>
<td></td>
<td>185-188</td>
</tr>
</tbody>
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Abbreviations: LPS, lipopolysaccharide; FCS, fetal calf serum; EGF, epidermal growth factor; LDL, low-density lipoprotein; PTH, parathyroid hormone.

Fig 1. CK-1 GGAGATTCACC (h/m IL-2, IL-3, GM-CSF, G-CSF). CK-2 TCAGGTA (h/m GM-CSF, IL-3). NF-B GGGAACCTACCC (non-coding strand GM-CSF).
human T-cell line. Furthermore, constructs prepared using an oligonucleotide containing CLE-2 and the GC-rich region (NF-κB site) driving a CAT reporter construct are able to be stimulated 10- to 80-fold by TPA and ionophore in Jurkat cells.27

NF-κB is a transcription factor that has been implicated in expression of a variety of genes induced upon T-cell activation.3-10 Work by Schreck and Baeuerle has shown that an NF-κB consensus sequence is present on the noncoding strand of the GM-CSF promoter.24 Using methylation interference analysis and oligonucleotide competition experiments, they demonstrated binding of purified NF-κB to this sequence, which overlaps the region containing CK-2 (CLE-2) and the GC-rich region described above. However, no functional data were presented to support the role of NF-κB in GM-CSF gene expression.

Our laboratory has taken a somewhat different approach by combining a biochemical and genetic analysis of the hGM-CSF promoter, using only human and primate cell lines and primary human cells. In experiments by Nimer et al, the upstream regions referred to as CK-1, CK-2, the GC-rich region, and the overlapping NK-κB region, are not required for expression of recombinant GM-CSF promoter constructs in stimulated T-cell lines, primary stimulated human T cells, or fibroblast cell lines (stimulated or unstimulated).3-24 Expression of recombinant constructs containing only 53 nucleotides (nt) upstream from the start site of transcription mimics either activation (T cells and T-cell lines) or constitutive expression (fibroblasts) of the endogenous gene.25-31 A repeated region located within 39 nt upstream of the TATA homology containing the core sequence, CATT(A/T), is essential for expression of recombinant constructs in all of the above cell types. Internal deletion or site-directed mutants that alter the CATT(A/T) repeats without altering 626 nt of upstream sequence destroy the activity of GM-CSF promoter constructs. In certain cell types, recombinant constructs containing only the −53 region show high basal activity, suggesting the importance of negative regulatory regions to inhibit transcription in unstimulated or inappropriate cell types (Fraser et al, submitted).35

DNase I footprinting studies also show the presence of a protein binding to this CATT(A/T) repeat in extracts from primary T cells stimulated with PHA, as well as stimulated T-cell lines (Fraser et al, submitted).35 An overlapping 14-nt sequence was also identified by Kausansky as being important in expression of GM-CSF recombinant constructs in endothelial and Jurkat cells.24 The region of the GM-CSF promoter that contains the CATT(A/T) repeats is highly conserved in the promoter of the IL-5 gene, which shows some overlapping biologic activities with GM-CSF on eosinophils, and is also expressed in activated lymphocytes.29

Human T-cell leukemia virus (HTLV)-transformed human lymphocytes have the phenotype of activated T cells, and express not only GM-CSF, but other lymphokines as well.29-34 HTLV type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL); HTLV type II (HTLV-II) has been associated with some specific T-cell disorders in humans.2 These retroviruses have two genes located in the 3' region of the genome that encode nonstructural proteins, Tax and Rex. A number of investigators have studied the constitutive expression of IL-2 and the IL-2 receptor gene in HTLV-transformed cells, and have come to the conclusion that the trans-activating proteins of HTLV-I or -II, p40Tα and p40Tβ, respectively, are capable of enhancing expression of the IL-2Ra gene through NF-κB binding sites.35-38

Studies by Miyatake et al implicate a region of the murine GM-CSF promoter overlapping the NF-κB region as being inducible by p40Tα in transient cotransfection experiments.39 However, Nimer et al show equally strong trans-activation of the −626 and −53 (which does not contain the NF-κB site) human GM-CSF promoter constructs by Tax in unstimulated T-cell lines.40 Trans-activation was totally abolished by either a nonsense mutation introduced into the Tax expression construct, or an internal deletion introduced into the GM-CSF promoter construct that removed the direct CATT(A/T) repeat without changing the NF-κB region. These results demonstrate that in the absence of T-cell activation, expression of Tax is sufficient to induce expression of recombinant GM-CSF promoter constructs in MLA 144 cells.

Perhaps the most interesting result is the cell type specificity of trans-activation by Tax. When Tax expression constructs were cotransfected with the HTLV long terminal repeat (LTR) into human B cells, CEM (an immature T-cell line), and COS cells, trans-activation was quite efficient.29 However, when the same constructs were cotransfected with the GM-CSF promoter constructs, there was no trans-activation. The results of these experiments show that, although HTLV-II Tax protein can trans-activate its own LTR in a variety of cell types, no activation of the GM-CSF CAT constructs is seen in cells that cannot be physiologically stimulated to produce GM-CSF. These results suggest that only mature T cells contain the factors required for trans-activation of the GM-CSF promoter by Tax.

The wide variety of approaches (band shift, footprinting, methylation interferences, and transient transfection) and cell types used make it difficult, at best, to reconcile the conflicting data on expression of GM-CSF in stimulated and HTLV-infected lymphocytes. It seems likely that both positive and negative regulatory elements act in a cell type-specific manner (see below) to regulate transcription of this gene. We believe that the two CATT(A/T) repeats located within the −53 promoter region are required for both inducible and constitutive expression of GM-CSF. Certainly other upstream protein-binding sites identified by biochemical assays could play roles in the stabilization or modification of transcription factor binding to the GM-CSF promoter.

The question of autocrine expression of GM-CSF in acute myeloid leukemia (AML) cells is one of great scientific interest and potential clinical importance. Because AML cells have GM-CSF receptors and are clearly responsive to the factor, it is of great importance to dissect the role of GM-CSF expression in the genesis or progres-
sion of myeloid leukemias. In an experimental model system, the factor-dependent myeloid cell line, D35, was found to become factor independent at a high frequency following retrovirus insertional mutagenesis. In 10 of 11 cases, factor-independent subclones were found to be secreting a growth stimulatory factor; six mutant clones expressed GM-CSF. Young et al found GM-CSF transcripts in a high percentage of AML cases studied, and recently proposed that regulation of GM-CSF expression is mediated primarily through posttranscriptional mechanisms, because nuclear run-on experiments demonstrated constitutive transcription of the gene in AML cells. In certain samples, the AML cells were cultured before extraction of RNA. Because it is known that GM-CSF expression in monocytes and macrophages can be stimulated by adherence to plastic or by the presence of fetal calf serum, it is unclear whether the patient’s AML cells were activated in vitro, or whether accumulation of GM-CSF RNA occurs in vivo. Others have suggested that spontaneous GM-CSF production occurs rarely in AML, and that the induction of IL-6 and GM-CSF gene expression is part of the normal differentiation program in myeloid cells.

We attempted to address this issue at the molecular level by studying the ability of recombinant GM-CSF promoter constructs to be expressed in AML cell lines. Fraser et al (manuscript submitted) demonstrated a high level of constitutive expression of recombinant constructs containing only 53 nt of upstream GM-CSF promoter sequences (containing two copies of the CATTA/T repeat) in M-07 and KG-1 cell lines. Addition of 16 nt upstream to −69 suppresses constitutive expression by threefold to 30-fold in myeloid leukemia cell lines. We used site-directed mutagenesis to identify sequences required for positive and negative activity in the −53 and −69 constructs, respectively. Our results demonstrate that the two direct CATTA/T repeats appear to be required for constitutive positive activity in AML cells, and that the negative regulatory region active in myeloid cell lines appears to involve a third CATTA/T direct repeat found within the −69 construct (see Fig. 1). Based on additional work using other constructs and other site-directed and spacing mutants, we developed a model in which the binding of activating factors to the positive regulatory domain may be obscured by binding of a repressor protein to an overlapping inverted repeat (manuscript submitted). Taken together, these studies predict that expression of the GM-CSF gene in AML cells will reflect the physiologic state of the cells that is determined by environmental stimuli and production of other cytokines.

PROTEIN STRUCTURE AND FUNCTION

Purification of GM-CSF from mouse lung conditioned medium was first reported by Burgess et al in 1977. Characterization of the purified material showed that GM-CSF was a glycoprotein with an apparent molecular weight of 23,000 to 29,000 d. However, the low yield of material prevented effective structural analysis and cloning of the molecule. Subsequently, treatment with neuraminidase greatly reduced the charge heterogeneity and allowed purification of a large amount of asialo GM-CSF. By this means, enough sequence information was obtained to isolate the cDNA clones encoding murine GM-CSF. In 1984, we purified hGM-CSF from medium conditioned by the HTLV-II–infected T-lymphoblast cell line, Mo. Purified hGM-CSF is also a glycoprotein with an apparent molecular weight of 22,000 d. Simultaneously, Wong et al, using a novel expression strategy, isolated a cDNA clone encoding hGM-CSF. They showed that the sequence of the cDNA clone encoded the natural GM-CSF protein that we and they had independently purified from medium conditioned by the Mo cell line. The human T-cell GM-CSF cDNA was found to be 60% homologous at the nucleotide level, with the sequence of the murine GM-CSF cDNA obtained using protein purified from mouse lung conditioned medium.

Human and murine GM-CSF cDNAs encode polypeptides of 144 and 141 amino acids, respectively. Both molecules contain 17 amino acid leader sequences and are heterogeneously glycosylated. Interestingly, removal of the carbohydrate, either by synthesis in bacteria or mutagenesis, actually increases the specific activity of GM-CSF. Two intrachain disulfide bonds are found in both murine and hGM-CSF. These disulfide structures are highly conserved, and are important for biologic activity. Despite 54% identity at the amino acid level, there is no cross-species reactivity between murine and hGM-CSF. Taking advantage of this observation, Kaushansky et al prepared chimeric mouse/human molecules to identify regions required for biologic activities. Two regions, one encompassing residues 21-31 (from the amino terminus of the mature protein), and the other encompassing residues 78-94, were found to be critical for function on hematopoietic cells. Clark-Lewis et al used a different strategy, and synthesized various fragments and analogues of hGM-CSF to determine regions required for biologic activity. A fragment containing residues 14-96 had detectable activity, yet, for full activity, residues 97-121 were also required.

Structure/function studies for murine GM-CSF were performed by in vitro mutagenesis, which showed critical residues within the regions 11-15, 24-37, 47-49, and 81-89. This result is in fairly good agreement with another study using scanning deletion analysis, and showed four critical regions spanning residues 18-21, 34-41, 52-61, and 94-115. The disulfide bridge between cysteines 51 and 93 was shown to be essential for activity; however, the bridge between cysteines 85 and 118 could be removed without loss of activity.

BIOLGIC ACTIVITIES OF GM-CSF

The purification, molecular cloning, and subsequent production of large quantities of biosynthetic (recombinant) hematopoietic growth factors has made it possible to study these factors in vitro and in vivo. During the past 5 years, there has been a virtual explosion of information in this field. While initial biologic characterizations were
performed in vitro with microgram quantities of purified protein, there is now a large body of information from clinical trials. We have gained a considerable amount of information that makes it possible to speculate upon the physiologic and pathophysiologic roles of these factors, yet much remains to be clarified.

GM-CSF acts as a potent growth factor both in vitro and in vivo, stimulating proliferation and maturation of myeloid progenitor cells, giving rise to neutrophilic and eosinophilic granulocytes and monocytes (see Table 2). Early in vitro experiments demonstrated that under certain culture conditions, GM-CSF can also stimulate proliferation of erythroid burst-forming units (BFU-E). In addition, there are reports that GM-CSF can stimulate proliferation or function of T-cell lines and plasmacytomas, although this remains somewhat controversial.

Early studies showed that purified murine GM-CSF and partially purified hGM-CSF not only act to stimulate proliferation of immature progenitors, but could also enhance differentiated functions of mature effector cells. Over the past 5 years, a long list of biologic activities of GM-CSF on mature neutrophils, monocytes, and eosinophils have been characterized (Table 2). Weisbart et al. first showed that purified human GM-CSF has both direct and indirect effects on human neutrophils. Direct effects include inhibition of neutrophil migration, degranulation, changes in receptor expression, and effects on cytoskeleton and cell shape. Indirect actions have been referred to as "priming effects" because they enhance the ability of the neutrophil to respond to a secondary triggering stimulus; among these effects are increased superoxide production, Ca2+ fluxes, and leukotriene B4 (LTB4) synthesis. The precise molecular and biochemical events involved in neutrophil priming have not been defined. It has been shown by several groups, including ours, that GM-CSF alters the number and affinity of f-Met-Leu-Phe (fMLP) receptors expressed on neutrophils. The changes in fMLP receptors correlate temporally with biologic responses. There is a rapid increase in receptor number that correlates with decreased neutrophil migration; the slower change in receptor affinity correlates with enhanced oxidative metabolism. The effects of GM-CSF on subsequent responsiveness to bacterial oligopeptides have also been described as recruitment of a less responsive subpopulation of cells, or potentiation of a normal neutrophil maturation process, making them more responsive to fMLP.

Another important priming or indirect effect of GM-CSF is its ability to enhance production of leukotrienes in neutrophilic and eosinophilic granulocytes. Production of these inflammatory mediators in response to chemotactic agents likely amplifies the inflammatory response. DiPersio et al. showed that GM-CSF has a small direct effect on neutrophil arachidonic acid release; it also directly stimulates production of low amounts of LTB4. Because neutrophils express LTB4 receptors and are downregulated by treatment with GM-CSF, it is possible that certain of the observed priming effects of GM-CSF are a result of autocrine stimulation of neutrophils by LTB4. This apparent autocrine production of LTB4 by GM-CSF-stimulated neutrophils complicates studies attempting to dissect biochemical signal transduction pathways mediating GM-CSF action (see below).

Many of the proposed therapeutic uses of GM-CSF and other hematopoietic growth factors center around their potential ability to augment current therapeutic regimens for cancer therapy and bone marrow transplantation. In addition to increasing the number of host defense cells, in vitro observations demonstrate that GM-CSF enhances the ability of neutrophils, macrophages, and eosinophils to phagocytize and destroy a variety of microorganisms.
Furthermore, GM-CSF increases antibody-dependent cell-mediated cytotoxicity (ADCC) towards tumor cells. Thus, it is hoped that GM-CSF treatment will not only protect the patient from the myelosuppressive effects of radiation and chemotherapy, but in the right setting, it may also augment the ability of the host defense system to locate and destroy tumor cells.

Pathophysiologic consequences of overproduction or inappropriate expression of GM-CSF have also been proposed. A potential autocrine role for GM-CSF in the establishment or progression of myeloid leukemias has been suggested (see above). In addition, the presence of GM-CSF biologic activity in synovial fluid from patients with rheumatoid arthritis suggests that it may enhance the tissue destruction associated with this disorder. Experimental overexpression of GM-CSF protein has been achieved using two strategies: establishment of transgenic mice, and insertion of retroviral vectors expressing GM-CSF into the bone marrow of mice. The transgenic mice exhibit blindness (caused by accumulation of macrophages in the eye) and infiltration of macrophages into striated muscles. Thus, the local presence of high concentrations of GM-CSF during development of the mouse embryo appears to be pathologic. Similarly, overexpression of GM-CSF in bone marrow cells infected with retroviruses carrying the murine GM-CSF cDNA leads to a myeloproliferative syndrome that is fatal, although non-neoplastic. Thus, expression of GM-CSF gene itself does not lead to neoplastic transformation as determined by subsequent transplantation of cells into normal mice. However, one could imagine that it may be a preliminary event leading to enhanced proliferation and more rapid accumulation of other genetic abnormalities in myeloid cells.

ACTIONS OF GM-CSF ON NONHEMATOPOIETIC CELLS

A number of reports have substantiated the GM-CSF responsiveness of various tumor cell lines of nonhematopoietic origin. Ruff et al first described responsiveness of small-cell carcinoma of the lung (SCCL) cell lines to GM-CSF. Baldwin et al went on to identify and characterize high-affinity GM-CSF receptors on SCCL cell lines. Work by Dedhar et al showed that hGM-CSF could stimulate proliferation of two osteogenic sarcoma cell lines, a breast carcinoma cell line, and an SV40-transformed marrow stromal cell line. Similarly, Berdell et al described the growth of colon adenocarcinoma cell lines in response to hGM-CSF. We were interested in determining whether fresh nonhematopoietic tumor cells express GM-CSF receptors, and whether those receptors might be functional. Having previously characterized GM-CSF receptors on small cell carcinoma of the lung cell lines, Baldwin et al looked at GM-CSF receptor expression of other tumors of neuroendocrine phenotype or neural crest origin, specifically malignant melanoma. We examined two melanoma cell lines and six fresh specimens obtained at the time of surgery and found expression of GM-CSF receptors on all fresh melanoma specimens and cell lines (see below) (Baldwin et al, manuscript submitted). However, the receptors observed on these cells are of lower affinity than those observed on responsive hematopoietic cell lines and small cell carcinoma of the lung (SCCL) cell lines. In addition, we were not able to demonstrate function or specific internalization of the ligand in melanoma cell lines. It will be of great interest to determine whether the normal counterparts of these neural crest-derived tumor cells also express GM-CSF receptors.

GM-CSF RECEPTORS

Considerable interest has been focused on molecular and biochemical characterization of the GM-CSF receptor in hopes that it will help to elucidate the mechanism by which this hematopoietin can regulate both proliferation and function in a wide variety of cell types. The murine GM-CSF receptor was first characterized by Walker and Burgess, who found specific binding of 125I-labeled GM-CSF to cells of the myelomonocytic lineage. Scatchard analysis of equilibrium binding data suggested that there were two classes of binding sites, one of high affinity (approximately 20 pmol/L), and the other of a lower affinity (approximately 1 nmol/L). DiPersio et al have extensively characterized the GM-CSF receptor on both proliferating and terminally differentiated human cells. Our data show that neutrophils possess a single class of high-affinity GM-CSF receptors (kd approximately 50 pmol/L), which are expressed in low numbers (approximately 800 to 1,000 sites/cell). Highest receptor expression is observed on the most differentiated hematopoietic cells, and receptor expression increases concomitantly with terminal differentiation in the HL-60 model system.

Our initial studies using the HL-60 cell line showed a low number of high-affinity binding sites with an additional component of binding (as analyzed by the Ligand program), which we then interpreted to be nonsaturable or low affinity. We have since characterized a low-affinity GM-CSF receptor on COS monkey cells, human melanoma cell lines, and primary melanoma cells (Baldwin et al, manuscript submitted). Chiba et al studied GM-CSF binding to monocytes, U937, and TF1 cell lines, and observed biphasic Scatchard estimating a high-affinity site with a kd of 10 to 40 pmol/L, and a low-affinity site with a kd of approximately 1 nmol/L. Park et al also described complex biphase curves for binding of GM-CSF to cells from patients with acute nonlymphocytic leukemia. These studies suggest that responsive cell types bind GM-CSF with high affinity; however, there is also evidence for low-affinity receptors on certain cell types.

DiPersio et al prepared membranes from a variety of cell types, and found that even when membranes were prepared from cells expressing a single class of high-affinity sites (such as neutrophils), the affinity of the GM-CSF receptor on membranes was decreased approximately one order of magnitude (kd increased from 50 pmol/L to 500 pmol/L). When membranes were solubilized and GM-CSF receptor binding was characterized in solution, this lower affinity interaction was maintained.

The internalization of GM-CSF receptors has been studied by Walker and Burgess, as well as by Nicola et al. Their experiments show that at 37°C the ligand-
bound GM-CSF receptor is rapidly internalized. It has also been suggested that unoccupied receptors are then recycled to the cell surface. Interestingly, several agents, including phorbol esters and the N-formylated oligopeptide, FMLP, downregulate neutrophil GM-CSF receptors. These observations led Cannistra et al to suggest that downregulation of GM-CSF receptors by certain activators of neutrophil function may negatively regulate their biologic effects.

Our crosslinking studies performed using a variety of cell types expressing either high- or low-affinity receptors show a major cross-linked species of approximately 97,000 d, which suggests a molecular weight of 84,000 d for the hGM-CSF receptor. Similar results on KG-1 cells have been observed by Gesner et al. Using chemical cross-linking, Chiba et al showed three major species of hGM-CSF receptor complexes at molecular masses of 150, 115, and 95 Kd. Park et al have identified a receptor protein using cross-linking of murine GM-CSF with an apparent molecular weight of 130,000 Kd. Recent molecular cloning of two components of the GM-CSF receptor helps to explain these apparent discrepancies.

Because it had been observed that placental membranes bind GM-CSF with low affinity, placental membranes provided a good source for the partial purification of the GM-CSF receptor, as well as molecular cloning of its cDNA. Last year, Gearing et al isolated two cDNA clones encoding a binding protein for hGM-CSF from a placental cDNA library. When the cDNA clone is transfected into COS cells, 125I-GM-CSF binds with low affinity (kd = 2 to 8 nmol/L). This finding is two orders of magnitude lower than the affinity estimated on whole cells such as neutrophils, and an order of magnitude lower than that observed on purified membranes. Cross-linking studies estimated a molecular weight of approximately 85,000 d, in good agreement with our cross-linking studies on a variety of cell types. The sequence of the cDNA clone predicts a large open reading frame of 400 amino acids, as well as certain structural features that have led Gearing et al and others to postulate the existence of a new receptor family.

This newly described receptor family includes the receptors for hGM-CSF, G-CSF, growth hormone, IL-3, IL-4, IL-6, IL-7, murine erythropoietin, the hIL-2 receptor beta-chain, and the rat prolactin receptor. None of these receptors contain a kinase domain; however, certain potentially important amino acid residues are conserved with this family. These residues include the positions of four cysteine residues and a region immediately extracellular of the transmembrane domain where there is a conserved sequence, tryptophan-serine-x-trypotphan-serine.

The biochemical relationship of low- and high-affinity of GM-CSF receptors is a perplexing topic. Using Scatchard analyses of equilibrium binding data, two classes of receptors have been described by a number of investigators. GM-CSF receptors characterized on purified membrane preparations and in solution are of low affinity. Reports from several laboratories have indicated that GM-CSF and IL-3 can cross-compete for binding on certain cell types (specifically, the KG-1 cell line, eosinophils, and primary acute nonlymphocytic leukemia (ANL) samples). Finally, the cloned receptor (isolated from placental membranes) transfected into COS cells exhibits binding of extremely low affinity (kd = 5 to 6 nmol/L). These observations have led us and others to postulate that the GM-CSF receptor is a multi-subunit complex. This hypothesis gains credence from the fact that other members of the newly described receptor family (specifically, IL-2 and IL-6 receptors) have also been shown to be multi-subunit complexes.

Loss of an associated subunit or so-called adaptor protein during the preparation of purified membranes could explain the reduction in binding affinity. The ability of GM-CSF and IL-3 to cross-compete for binding on cell types expressing both receptors could be due to their ability to share certain subunits or adaptor proteins.

Recent work from Hayashida et al helps to explain several of these puzzling questions. They isolated the human homologue of the mouse IL-3 receptor cDNA, which is a transmembrane protein of 120 Kd. Cells transfected with the human cDNA alone did not bind either hIL-3 or GM-CSF; however, when cotransfected with the low-affinity GM-CSF receptor, a high-affinity receptor for GM-CSF was formed. Their cross-linking studies showed that 125I-GM-CSF could bind to both the 80-Kd and 120-Kd subunits, which is consistent with cross-linking data obtained by binding to certain cell types.

A critical question then becomes whether the 84,000-d low-affinity GM-CSF binding protein is functional, or if additional subunits are required for transduction of an intracellular signal. Using retroviral vectors to introduce the hGM-CSF receptor cDNA into a factor-dependent murine cell line, Metcalf et al recently reported that the hGM-CSF receptor can deliver a proliferative signal. Their results showed that concentrations of GM-CSF required to stimulate proliferation through the human (recombinant) receptor were 500 to 1,000 times greater than the concentrations of murine GM-CSF required to elicit a biologic response. In addition, internalization studies showed that while the 125I-GM-CSF associated rapidly with the cell surface, it was slowly internalized. Thus, while no high-affinity binding was detected, a proliferative response could be elicited by high concentrations of GM-CSF, suggesting that the low-affinity receptor is capable of transducing a functional signal, possibly through association with an adaptor subunit constitutively expressed in the murine cell line.

Baldwin et al studied naturally occurring low-affinity GM-CSF receptors expressed on melanoma cells, which are of nonhematopoietic origin (Baldwin et al, manuscript submitted). Melanoma cell lines and several fresh melanoma specimens were shown to possess approximately 500 GM-CSF receptors/cell, with a kd of approximately 500 pmol/L (10-fold lower than the kd of receptors expressed on responsive hematopoietic cells and cell lines). We found no induction of primary response genes and no proliferative response in melanoma cells treated with GM-CSF. Our results did not support the conclusion that these low-affinity receptors can transduce a signal. In addition, internalization studies comparing internalization of high- and low-
affinity receptors after ligand binding showed that greater than 90% of the ligand internalized in cells expressing high-affinity receptors was specifically bound, whereas less than 10% of $^{125}$I-GM-CSF internalized by cells expressing only low-affinity receptors was specifically bound.

The low-affinity GM-CSF receptor cDNA clone was used by Gough et al to localize the hGM-CSF receptor to the X-Y pseudoautosomal region. Alleles in this region can be exchanged between X and Y chromosomes, and are, therefore, inherited as if autosomal. It is postulated by the authors that loss of this gene may be important in understanding the generation of the M2 subclass of acute myeloid leukemias, because loss or inactivation of both copies of the GM-CSF receptor could result in generation of a relatively immature cell incapable of responding to GM-CSF. Reportedly 25% of the M2 subtype of AMLs have lost either the X or Y chromosome.

**GM-CSF SIGNAL TRANSDUCTION**

As described above, molecular analysis of the low-affinity GM-CSF binding subunit did not show specific signal transduction pathways. It has been reported that GM-CSF stimulates phosphorylation of proteins on serine and tyrosine residues. Because the GM-CSF binding subunit does not contain kinase activity, other subunits associated with the complex may be kinases, or lead to production of second messengers that activate cellular kinase systems.

Perhaps the most confusing issue has been the involvement of guanine nucleotide-binding proteins in GM-CSF action. Several groups have reported that certain biologic activities induced by GM-CSF treatment of neutrophils can be inhibited by pertussis toxin (PT), implying that a PT-sensitive G-protein was important in mediating GM-CSF action. However, we have shown additional effects of GM-CSF on neutrophils that could not be inhibited by PT. Because the GM-CSF binding subunit does not contain kinase activity, other subunits associated with the complex may be kinases, or lead to production of second messengers that activate cellular kinase systems.

Many tools are now available to further dissect the regulation of GM-CSF expression and its mechanism of action. There are still critical gaps in our understanding that prevent us from putting together a comprehensive picture of the molecular physiology of GM-CSF. While we can speculate on the role of GM-CSF in host defense, we know much less about its potential role (if, in fact, it plays a role at all) in homeostatic maintenance of myelopoiesis. Although the GM-CSF receptor binding subunit has recently been characterized, much remains to be determined about signal transduction pathways, as well as other subunits that constitute the fully functional GM-CSF receptor complex. However, because of the large number of responsive cell lines and the ready availability of normal bone marrow and peripheral blood cells, it seems likely that further studies on the mechanism of action of GM-CSF and other hematopoietins will help to shed light on more general questions involving cell proliferation and transformation.

As our understanding of the molecular physiology of individual hematopoietins grows, we must aim to integrate that knowledge into processes occurring in the whole organism, as well as define the role of each cytokine in relation to other hematopoietins with overlapping biologic activities. With the current availability of reagents and technology, it seems likely that our understanding of the regulation of hematopoiesis and the role of GM-CSF in blood cell production and function will continue to grow and provide biologic insights leading to new therapeutic applications.

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**REFERENCES**


39. Chan JY, Slamon DJ, Nimer SD, Golde DW, Gasson JC:
41. Chen ISY, Quan SG, Golde DW: Human T-cell leukemia virus type II transforms normal human lymphocytes. Proc Natl Acad Sci USA 80:7006, 1983
45. Begley CG, Metcalf D, Nicola NA: Primary human myeloid leukemia cells: Comparative responsiveness to proliferative stimulation by GM-CSF or G-CSF and membrane expression of CSF receptors. Leukemia 1:1, 1987
57. Shabo Y, Lotem J, Sachs L: Regulation of the genes for interleukin-6 and granulocyte-macrophage colony stimulating factor by different inducers of differentiation in myeloid leukemic cells. Leukemia 3:859, 1989
64. De Lamarter JF, Mermod J-J, Liang C-M, Eliason JF, Thatcher DR: Recombinant murine GM-CSF from E. coli has biological activity and is neutralized by a specific antisera. EMBO J 4:275, 1985
72. Tomonaga M, Golde DW, Gasson JC: Biosynthetic (recombinant) human granulocyte-macrophage colony-stimulating factor:
Effect on normal bone marrow and leukemia cell lines. Blood 67:31, 1986


104. Nathan CF: Respiratory burst in adherent human neutro-


136. Nicola NA, Peterson L, Hilton DJ, Metcalf D: Cellular processing of murine colony-stimulating factor (multi-CSF, GM-
CSF, G-CSF) receptors by normal hemopoietic cells and cell lines.

Growth Factors 1:41, 1988


144. Onetto-Potier N, Aumont N, Hamon A, Park L, Clark SC, De Lean A, Hoang T: IL-3 inhibits the binding of GM-CSF to AML blasts, but the two cytokines act synergistically in supporting blast proliferation. Leukemia 4:329, 1990


168. Wodnar-Filipowicz A, Heusser CH, Moroni C: Production of the haemopoietic growth factors GM-CSF and interleukin-3 by...


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JC Gasson