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**REVIEW ARTICLE**

## Granulocyte Colony-Stimulating Factor and Its Receptor

By George D. Demetri and James D. Griffin

**G**RANULOCYTE colony-stimulating factor (G-CSF) is a polypeptide growth factor that regulates the production of neutrophilic granulocytes. This physiologic process serves as the foundation for critical host defense systems and occurs on a large scale in vivo. An adult of average size will produce approximately 120 billion granulocytes per day simply to replace normal losses. This enormous production capacity may be increased by at least 10-fold under stress conditions such as infection. G-CSF is likely to play a role in the basal regulation of neutrophil production as well as to function as a primary regulatory factor controlling the neutrophil response to inflammatory stimuli. Further, G-CSF exhibits other biologic activities besides proliferative effects: specifically, G-CSF appears to modulate certain neutrophil functions as well as the distribution of neutrophils and progenitor cells within the body. G-CSF possesses unique and interesting characteristics among the family of hematopoietic growth factors. This review will summarize the current state of knowledge of the structure and function of G-CSF and its receptor.

### IDENTIFICATION OF G-CSF

The identification of CSFs was made possible by the cell culture assays for hematopoietic progenitor cells, which were developed in the mid 1960s independently by Metcalf, Sachs, and their colleagues. These in vitro systems showed that the survival, proliferation, and differentiation of immature hematopoietic cells were dependent on the continued presence of humoral factors, which were collectively termed "colony-stimulating activity" (CSA). Before the purification of individual factors, early sources of CSA included media conditioned by culture with stimulated normal blood or splenic leukocytes, placenta, or certain tumor cells.<sup>1-5</sup> Also, serum collected after administration of endotoxin to animals was shown to contain CSA. It was initially unclear whether the complex mixtures termed CSA contained individual factors specific for proliferation and separate factors that specifically induced differentiation. Depending on the assay system used, various biologic activities were discovered and characterized. Further investigation showed that many of these biologic activities were attributable to the simultaneous presence of multiple factors in the crude CSA. Purification of the CSFs proved difficult, and, for many factors, expression cloning and production of recom-

binant protein were required to completely define the unique biologic properties of individual CSFs.

The nomenclature applied to partially purified CSFs can be confusing. G-CSF was probably first identified as a distinct activity by Burgess and Metcalf, not by its ability to stimulate proliferation, but rather by the capacity of postendotoxin-treated mouse serum or conditioned media to induce differentiation of a murine myelomonocytic leukemia cell line, the differentiation-responsive (D<sup>+</sup>) subline of WEHI-3B cells.<sup>6,7</sup> Therefore, G-CSF was initially termed a granulocyte-macrophage differentiation factor (GM-DF) by Metcalf<sup>7</sup> and was noted to be related to (or the same as) a differentiating activity named MGI-1G by Lotem et al.<sup>8</sup> G-CSF was shown to be separate from GM-CSF, which had been partially purified in the late 1970s. This distinction was experimentally determined by the generation of neutralizing antisera that could block the effects of GM-CSF but which failed to block the activity of GM-DF. GM-DF was then shown to copurify with a novel activity that selectively stimulated the formation of granulocytic colonies by normal hematopoietic progenitor cells in vitro,<sup>7</sup> and after further purification, this factor was ultimately renamed G-CSF.<sup>9</sup> Nicola et al<sup>9</sup> described the biochemical characteristics of murine G-CSF in 1983 as a hydrophobic glycoprotein with an apparent molecular weight of 24 or 25 Kd, containing a neuraminic acid moiety and at least one internal disulfide bond necessary for biologic activity.

After the identification of the murine G-CSF, a human molecule with analogous activities was discovered. On the basis of biologic actions and receptor binding studies, human G-CSF was shown to be the same as "CSF- $\beta$ ," a hematopoietic stimulatory activity previously identified in human placenta-conditioned medium.<sup>10</sup> Both the proliferation- and differentiation-inducing activities of the murine and human G-CSF molecules crossed species boundaries, in contrast to other hematopoietic growth factors such as

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GM-CSF or interleukin-3 (IL-3), which are active on the neutrophilic lineage in a species-specific manner. At the same time that human G-CSF was identified as CSF- $\beta$ , Welte et al reported the purification to homogeneity of an activity designated "human pluripotent hematopoietic colony-stimulating factor"<sup>11</sup> or "pluripoietin,"<sup>12</sup> a CSF that was constitutively secreted into culture medium by the human bladder carcinoma cell line 5637. Although this tumor cell-derived CSF was reported to induce differentiation of both murine WEHI-3B(D<sup>+</sup>) and human HL-60 leukemic cells, it was initially felt that this did not represent the human G-CSF because it appeared to stimulate growth of multilineage (CFU-GEMM) and early erythroid (BFU-E) colonies from normal human bone marrow.<sup>11,12</sup> With further study, it became clear that the putative multilineage stimulatory activity was an indirect effect and that the primary activity of purified native pluripoietin was to induce formation of colonies of neutrophilic granulocytes in vitro.<sup>13</sup> The identity of pluripoietin as human G-CSF was confirmed by sequence analysis<sup>14</sup> as well as by direct comparison with the biologic activity of recombinant G-CSF.<sup>13</sup> The production of G-CSF by human nonhematopoietic tumor cell lines has been shown to be relatively common, and other investigators reported the production of G-CSF by the squamous cell carcinoma cell line CHU-2<sup>15</sup> and by the hepatoma cell line SK-HEP-1.<sup>16</sup> Nomura et al<sup>15</sup> purified native human G-CSF from culture medium conditioned by the tumor cell line CHU-2 and described its properties as a molecule that specifically induced the growth of cells of the neutrophilic granulocyte lineage.<sup>15</sup>

#### CLONING OF THE G-CSF GENE

Further understanding of the biologic and biochemical properties of G-CSF was greatly facilitated by cloning of the gene encoding G-CSF and the production of recombinant protein for study. In 1986, Souza et al<sup>14</sup> reported the cloning of a cDNA encoding human G-CSF from a cDNA library derived from the tumor cell line 5637, using degenerate oligonucleotide probes generated from partial N-terminal sequencing of native human G-CSF purified from 5637 bladder carcinoma cells. The properties of the recombinant human protein were shown to be very similar to those of native murine G-CSF.<sup>14</sup> Using a similar strategy, Nagata et al<sup>17</sup> cloned a cDNA for G-CSF from the squamous cell carcinoma cell line CHU-2. The cDNA clones obtained by these two groups differed: the clone derived by Souza et al encoded a protein of 174 amino acids, while the cDNA obtained by Nagata et al encoded a larger protein of 177 amino acids. The larger protein was significantly less active at stimulating proliferation of progenitor cells than the smaller one. Subsequent studies by Nagata et al showed that a second cDNA encoding the 174-amino acid G-CSF could also be derived from the CHU-2 tumor cell line. This smaller G-CSF molecule has a deletion of 3 amino acids (Val-Ser-Glu) between Leu<sup>35</sup> and Val<sup>36</sup> at the amino terminus of the larger G-CSF.<sup>18</sup> The significance of the two G-CSF RNA species in the CHU-2 tumor cell line remains unclear. Other data also suggest that the amino terminus plays an important role in the function of G-CSF. In a study

designed to analyze the relationship of G-CSF structure to biologic activity of the molecule, Okabe et al<sup>19</sup> generated different mutant forms of recombinant human G-CSF protein that were assayed for hematopoietic activity. One mutant G-CSF with five amino acid substitutions in the amino terminus of the molecule exhibited enhanced in vitro and in vivo hematopoietic activity, possibly due to increased stability of this mutant G-CSF compared with wild-type G-CSF with native sequence.<sup>19</sup> This study did not compare the activity of the mutated recombinant G-CSF with purified native human G-CSF, so it is possible that the differences noted between these molecular forms of G-CSF might be due to a relative lack of activity of this particular recombinant G-CSF molecule, rather than an enhanced activity of the molecule with the amino terminal modifications.

Southern blot analysis of genomic human DNA showed that human G-CSF is encoded by a single gene,<sup>17,18</sup> and further studies determined that this single gene is located on chromosome 17 q11-22.<sup>20,21</sup> The genomic structure of the human G-CSF gene was determined by Nagata et al.<sup>18</sup> The G-CSF gene consists of 5 exons spread over a locus of approximately 2.3 kb. At the 5'-terminus of the second intron, two donor splice sequences are present in tandem, only 9 bp apart. The two different G-CSF cDNAs found in the CHU-2 cell line are produced by alternative splicing using one of these two donor sites and a common splice acceptor site within intron 2.<sup>18</sup> The molecular mechanisms that dictate the choice of donor splice site used (and thus the size of the G-CSF molecule ultimately produced) remain unknown.

The chromosomal localization of the G-CSF gene to chromosome 17 at 17 q11-22 initially raised questions about whether the G-CSF gene might be involved in the breakpoints of the t(15;17) translocation characteristic of acute promyelocytic leukemia (APL). However, the G-CSF gene is located proximal to the breakpoint of the t(15;17) translocation and is not rearranged in the malignant clone that gives rise to APL.<sup>20,21</sup> The localization of human G-CSF on chromosome 17 differs from that of several other human hematopoietic growth factors such as GM-CSF, IL-3, IL-4, and IL-5, which are clustered on the long arm of chromosome 5.<sup>22</sup> The murine G-CSF gene is located on chromosome 11 in a region that is homologous to human chromosome 17. The genes encoding GM-CSF and IL-3 are also located on murine chromosome 11, but within a separate region that is homologous to the human chromosome 5.

Following the description of the cDNA for human G-CSF, the murine G-CSF gene was cloned by cross-hybridization with a human G-CSF cDNA probe under low stringency conditions.<sup>23</sup> The murine G-CSF gene is highly homologous with the human gene, with 69% nucleic acid sequence homology in both coding and noncoding regions, and a 73% sequence homology in the predicted amino acid sequence of the protein.<sup>23</sup> Four of the five cysteine residues in both the murine and human G-CSF proteins are located within conserved positions, consistent with the previous data that G-CSF contains internal disulfide bonds which are necessary for maintenance of tertiary structure and

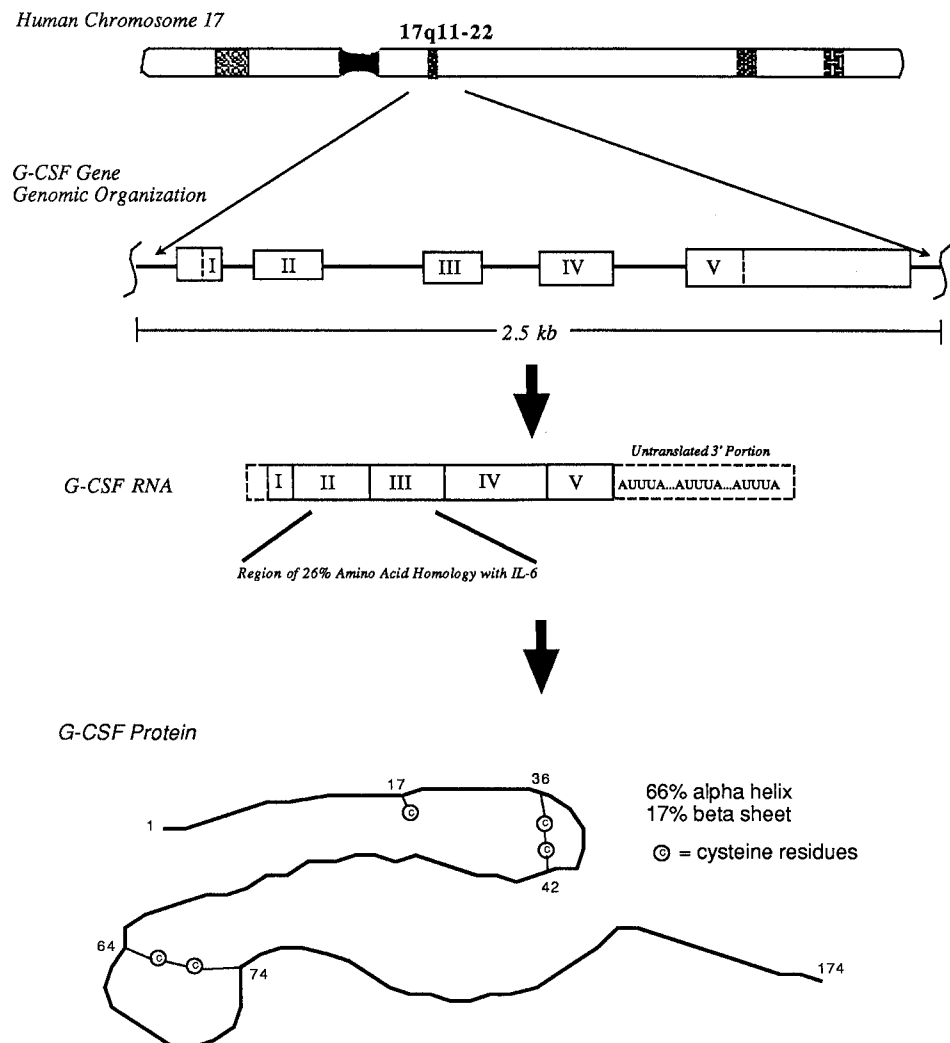
biologic activity.<sup>9</sup> The cysteine residue at position 17 has been shown to exist in the reduced form in a biologically active recombinant human G-CSF molecule and does not participate in intraprotein disulfide bridging. Further, substitution of Cys<sup>17</sup> by a serine residue does not adversely affect biologic activity, and appears not to alter protein conformation significantly.<sup>24</sup> The genomic organization of the murine G-CSF gene is very similar to the human G-CSF gene as well, consisting of 5 exons spanning 2 kb (see Fig 1). Interestingly, there is no tandem repeat of a donor splice site in intron 2 of the murine gene, and no alternative splicing occurs in processing of the murine G-CSF mRNA.<sup>25</sup>

The human G-CSF gene is distantly related to the IL-6 gene. The number, location, and size of the introns and exons that comprise these two genes are similar. Additionally, the amino acid sequences of G-CSF and IL-6 share some localized homology. Between amino acid residues 20 to 85 of G-CSF, the positions of 17 residues match with residues located between positions 28 to 91 of the IL-6 molecule, which yields a sequence homology for this region of 26%.<sup>26,27</sup> Additionally, the positions of four cysteine residues are precisely conserved between G-CSF and IL-6

in this region of relative homology. The tertiary structure of G-CSF may be quite similar to that of IL-6, particularly if intrachain disulfide bridges are similarly located within these molecules. Thus, it is possible that the genes encoding G-CSF and IL-6 may have arisen from a gene duplication event from which they have subsequently diverged. There is no linkage of chromosomal localization between G-CSF and IL-6 because the human IL-6 gene is located at chromosome 7p15.<sup>28</sup>

#### REGULATION OF G-CSF GENE EXPRESSION

There is a marked diversity in both the types and locations of cells capable of producing G-CSF. Given this diversity as well as the variety of stimuli that elicit an increase in G-CSF production, it could be anticipated that the control of G-CSF gene expression is likely to be complex. In particular, it is likely that G-CSF gene expression will be regulated differently in different cell types. The expression of most cytokine genes is regulated by a combination of transcriptional and posttranscriptional mechanisms,<sup>29</sup> and both types of regulatory mechanisms have



**Fig 1. Molecular organization of the gene encoding human G-CSF, structure of the G-CSF RNA transcript, and secondary structure of the native human G-CSF protein.**

been shown to be relevant for the control of G-CSF gene expression (Table 1). The 300 nucleotides upstream of both the murine and human transcription initiation codon for the G-CSF genes are highly conserved and are likely to contain important promoter elements.<sup>25</sup> Based on a CAT assay system to analyze transcriptional activity in deletion mutants of the G-CSF promoter, Nishizawa et al<sup>30,31</sup> have determined that three *cis* regulatory elements are required for the endotoxin-mediated induction of G-CSF by murine monocyte/macrophage cell lines or for the constitutive expression of G-CSF by carcinoma cell lines. Three specific sites have been identified as candidates for regulatory elements within this conserved upstream region. An octamer sequence (ATTTGCAT) located 110 bp upstream of the TATA box may regulate transcription via interaction with an octamer-binding factor, analogous to the octamer sequence in the Ig gene promoter that can interact with the B-cell specific transcriptional activator OTF-2.<sup>32</sup> Another possible *cis* regulatory element is the decanucleotide sequence (GAGATCCCC) located approximately 180 bp upstream of the major transcription start site. This decanucleotide sequence, conserved in sequence and genomic location between the murine and human G-CSF genes, bears a strong resemblance to the consensus sequence for the NF- $\kappa$ B transcriptional factor (GGGGGATTCC).<sup>33,34</sup> Additionally, this decanucleotide sequence is highly conserved in sequence (although not in genomic location relative to the TATA boxes) within the regions upstream of the genes encoding G-CSF, GM-CSF, and IL-3. This conservation within the likely promoter elements of several cytokine genes has led to the designation of this sequence as either the "CSF box,"<sup>30,31</sup> the "cytokine consensus element,"<sup>35,36</sup> or the "conserved lymphokine element 1 (CLE-1)."<sup>37</sup> The physiologic significance of this conserved element remains controversial, because there are data which suggest that nuclear protein factors isolated from macrophages do not bind to this decanucleotide sequence directly, but rather to a separate region located somewhat closer to the coding sequence.<sup>30</sup> The third suspected regulatory element in the promoter of the G-CSF gene is a sequence approximately 40 nucleotides long that bears no resemblance to known promoter elements from other genes.

While these data support the notion that transcriptional activation may play a part in increasing G-CSF gene expression after stimulation, other lines of evidence implicate posttranscriptional mechanisms as also being important in this process. Several reports have documented that certain cytokine genes, including G-CSF, are constitutively transcribed by a variety of cell types *in vitro*, including blood monocytes, fibroblasts, and mesothelial cells.<sup>38-40</sup> In general, the G-CSF mRNA produced by these cells is short-lived, with a message half-life less than 15 minutes.<sup>38,40</sup> The mRNA that encodes G-CSF contains poly-AUUUA sequences in the 3'-untranslated region which have been associated with mRNA instability.<sup>41</sup> These poly-AUUUA sequences have also been associated with mRNA instability.<sup>41</sup> These poly-AUUUA sequences have also been found in the mRNAs encoding other cytokines, including GM-CSF, IL-1, IL-6, certain interferons, and tumor necrosis factor (TNF), as well as in several cell growth regulatory genes (including the retinoblastoma susceptibility gene Rb-1, and such proto-oncogenes as *c-jun*, *c-fos*, *c-myc*, and *c-myb*). After exposure of fresh human monocytes to lipopolysaccharide, the half-life of G-CSF mRNA increases transiently.<sup>39</sup> This increase in message stability results in an accumulation of G-CSF mRNA, which then becomes detectable by Northern blot analysis. Other agents that increase the stability of G-CSF mRNA include IL-1, TNF, phorbol myristate acetate, and cycloheximide.<sup>38,40</sup> It has been suggested that these agents interfere with an RNase activity that may recognize the AUUUA-rich region of cytokine transcripts and that may therefore play a role in coordinate regulation of several cytokine genes.<sup>42</sup> The activity of cycloheximide, an agent that blocks new protein synthesis, in inducing increased levels of G-CSF mRNA may be explained if this putative RNase is a short-lived protein that requires constant replenishment by cellular protein synthesis. It is notable that a protein factor has been identified which appears to bind to this AUUUA "cytokine stability consensus sequence."<sup>43</sup> Increased levels of G-CSF mRNA transcripts secondary to enhanced stability have also been noted in certain cells that express mutant *ras* oncogenes.<sup>40</sup> The aberrant production of significant amounts of G-CSF by certain tumor cells may stem from abnormal processing of G-CSF mRNA and increased mRNA accumulation.<sup>44,45</sup> The molecular mechanisms responsible for these changes in mRNA stability and accumulation remain poorly defined. The induction of G-CSF gene expression in normal cells after stimulation is a transient phenomenon, with a gradual diminution in gene expression occurring within 8 to 12 hours after the peak even in the continued presence of the inducing agent. These kinetics for induction and suppression of G-CSF gene expression do not apply to tumor cell lines that constitutively produce G-CSF, because these cells appear to lack the normal regulatory pathways that confer short-message half-life to the G-CSF transcripts. The relative importance of transcriptional mechanisms versus posttranscriptional mechanisms in the physiologic control of G-CSF gene expression are not known, and these may vary among cell types. For example, no increase in transcriptional activity of the G-CSF gene was noted in fresh human

**Table 1. Regulation of G-CSF Production**

<b>I. Transcriptional mechanisms</b>	
Upstream elements possess similarities to known classes of regulatory elements	
Octamer sequence	
Decanucleotide sequence	
<b>II. Posttranscriptional mechanisms</b>	
Constitutive transcription results in G-CSF mRNA with short half-life	
Stability of G-CSF mRNA increases transiently following inducing stimuli (eg, IL-1, PMA, LPS, cycloheximide)	
Constitutive production of abnormally stable G-CSF mRNA is associated with certain tumor cells and expression of <i>ras</i> mutants	



monocytes after lipopolysaccharide (LPS) stimulation,<sup>39</sup> although an increase in transcription was observed in LPS-stimulated murine monocyte cell lines.<sup>30</sup> In fibroblasts exposed to TNF- $\alpha$ , the increase in G-CSF gene expression is mediated by both transcriptional activation and increased mRNA stability.<sup>38</sup>

Another important aspect of cytokine gene regulation is whether G-CSF is regulated coordinately or independently with regard to other cytokine genes in the same cells. In human blood monocytes, the G-CSF gene is regulated independently of the M-CSF gene, and exposure of these cells to different culture conditions can elicit selective expression of these two cytokines.<sup>39,46</sup> However, in mesenchymal cells such as fibroblasts and mesothelial cells, the G-CSF gene is clearly regulated in a coordinate manner with other genes encoding cytokines such as GM-CSF and IL-6<sup>38,40,44</sup>, ie, these genes are all induced by the same stimuli with similar kinetics. A more complete understanding of the molecular regulatory pathways responsible for G-CSF gene expression against the background of gene expression of other cytokines will be useful for dissecting out the various stimuli that control hematopoiesis, as well as those that mediate host defense via inflammatory pathways and modulation of effector cell function.

#### PRODUCTION OF G-CSF: CELLULAR SOURCES AND POSSIBLE PHYSIOLOGIC ROLES

The ability to produce G-CSF is characteristic of a variety of cell types following appropriate stimulation. Cells of the monocyte/macrophage lineage are among the most prominent sources of G-CSF, but this factor can also be produced by normal cells of mesodermal origin, including vascular endothelial cells,<sup>47</sup> fibroblasts,<sup>48</sup> and mesothelial cells.<sup>44</sup> Production of G-CSF can be induced *in vitro* in these cells by a wide variety of stimulatory agents, including LPS,<sup>39,46</sup> TNF,<sup>48</sup> IL-1,<sup>47,49</sup> 12-*o*-tetradecanoylphorbol-13-acetate (TPA),<sup>38,44</sup> GM-CSF,<sup>46,50</sup> IL-3,<sup>50</sup> IL-4,<sup>51</sup> and interferon- $\gamma$ .<sup>52</sup>

In virtually all normal cells examined to date, production of the G-CSF protein is highly regulated and not constitutive. As noted previously, constitutive transcription of the G-CSF gene does not necessarily imply continuous production of detectable levels of G-CSF protein. In contrast, malignant cell lines derived from numerous cell types may constitutively secrete large amounts of G-CSF. Examples of nonhematopoietic tumor cell lines that aberrantly produce G-CSF in a constitutive manner include lines obtained from bladder carcinoma,<sup>11</sup> hepatoma,<sup>16</sup> squamous cell carcinoma of the oropharynx,<sup>17</sup> melanoma,<sup>53</sup> mesothelioma,<sup>44</sup> sarcoma,<sup>23,54</sup> and glioblastoma.<sup>55</sup> Aberrant production of G-CSF by tumor cells may play an important etiologic role in the paraneoplastic leukemoid reactions that are occasionally seen in association with certain solid tumors.

The physiologic control of G-CSF production *in vivo* remains incompletely understood. In particular, it is unclear what role is played by G-CSF in maintenance of normal steady-state hematopoiesis. The levels of circulating G-CSF in normal individuals and in patients with a variety of diseases have been analyzed using either a bioassay specific for G-CSF<sup>56</sup> or enzyme-linked immunoassays.<sup>57,58</sup> In normal humans, the circulating levels of G-CSF are gener-

ally below the limits of detection of even the most sensitive immunoassays (ie, < 30 pg/mL) and, even when detectable, have always been < 100 pg/mL.<sup>57,58</sup> However, under stress conditions such as infection or following high-dose cytotoxic therapy with bone marrow transplantation, G-CSF levels increase dramatically and may exceed 2,000 pg/mL.<sup>57</sup> Further, after recovery from cytotoxic chemotherapy-induced myelosuppression, there tends to be an inverse relationship between endogenous G-CSF serum levels and the circulating neutrophil counts: the highest levels of G-CSF are attained during the neutrophil nadir, and levels decrease as the neutrophil count increases. Circulating levels of G-CSF have also been noted to increase during the neutropenic phase of human cyclic neutropenia.<sup>57</sup> These data suggest that G-CSF may function as a systemic regulator of the level of circulating neutrophils in normal individuals under this stress condition. It remains uncertain as to whether G-CSF regulates the numbers of neutrophils in basal hematopoiesis, although there are some data in a canine model that support this theory. Normal dogs treated with human G-CSF may develop neutralizing antibodies to the human molecule that can cross-react with endogenous canine G-CSF. Dogs that develop these anti-G-CSF antibodies exhibit a profound neutropenia which persists as long as the antibodies are present, suggesting that G-CSF is critical to the maintenance of normal neutrophil numbers.<sup>59</sup> No correlative human data exist because no anti-G-CSF neutralizing antibodies have yet been reported in patients with neutropenic states or in patients being treated with recombinant human G-CSF. These results, although not definitive, suggest that circulating G-CSF may be important as a physiologic mediator which the body uses to maintain an appropriate level of peripheral neutrophils. Thus, it could be predicted that there is a physiologic "neurostat" which would somehow sense the number of peripheral neutrophils and set the level of G-CSF necessary to maintain, decrease, or increase the population of mature neutrophils, in much the same way as oxygen-sensing cells in the kidney regulate erythropoietin production. Alternatively, it may be that the role of G-CSF in basal hematopoiesis is minimal or merely permissive and that it may not function as a primary regulator of neutrophil production *in vivo*. If this were to be the case, G-CSF might function primarily as an emergency signal to increase neutrophil production under conditions of stress. It remains an intriguing biologic puzzle to consider how the production of G-CSF is regulated from a systemic level, considering the wide variety and distribution of cells capable of G-CSF synthesis.

#### THE RECEPTOR FOR G-CSF: IDENTIFICATION, BIOLOGIC CHARACTERISTICS, AND MOLECULAR CLONING

G-CSF, like each of the other CSFs and ILs, exerts its biologic activities through binding to CSF-specific, high-affinity receptors. Nicola and Metcalf<sup>60,61</sup> studied the cellular binding of a biologically active, radioiodinated species of G-CSF to murine hematopoietic cells and demonstrated the existence of specific, saturable, high-affinity receptors for G-CSF on cells of the neutrophilic granulocyte lineage.

G-CSF receptors were noted to be present on cells from the myeloblast to the mature neutrophil, but not on any erythroid or megakaryocytic lineage cells.<sup>61</sup> Interestingly, a subset of monocytic cells was noted to bind G-CSF at very low levels.<sup>61</sup> The number and affinity of G-CSF receptors on normal murine marrow cells were similar to the receptors identified on the surface of the murine leukemic cell line WEHI-3B D<sup>+</sup>.<sup>60,61</sup> Mature neutrophils had the greatest numbers of G-CSF receptors per cell, and, in general, the receptor number increased with maturation level, with neutrophils exhibiting twofold to threefold more receptors than band forms or metamyelocytes. However, even neutrophils expressed a relatively low number of G-CSF receptors (approximately 50 to 500 receptors per cell).<sup>61</sup> An apparent kinetic dissociation constant (kd) of 65 to 90 pmol/L at 37°C was found on normal marrow cells and on WEHI-3B D<sup>+</sup> cells. The fact that half-maximal biologic activity of G-CSF occurs at a significantly lower concentration (3 pmol/L) suggests that occupancy of only a small fraction of the total number of G-CSF surface receptors may be sufficient to stimulate maximal biologic response.<sup>61</sup> The biologic phenotype of the murine leukemia cell subline WEHI-3B D<sup>-</sup>, which fails to differentiate in response to G-CSF, was also explained by the study of the G-CSF receptor, because this subline failed to exhibit any specific binding of G-CSF and appears to lack a functional cell-surface G-CSF receptor.<sup>60</sup>

Relatively little is known at this time about the regulation of G-CSF receptor expression. In murine marrow cells, there is a hierarchical regulation of receptors for hematopoietic growth factors such that the G-CSF receptor can be "down-modulated" by prior exposure of cells to GM-CSF, IL-3, or G-CSF itself. This "cross-modulation" of hematopoietin receptors involves undefined mechanisms of receptor signaling because neither GM-CSF nor IL-3 compete directly for G-CSF ligand binding sites on these cells.<sup>62</sup> Human neutrophils have also been shown to possess high-affinity, saturable binding sites using radiolabeled murine G-CSF as a ligand, with an apparent kinetic kd of approximately 900 pmol/L at 37°C and with a range of 700 to 1,500 receptors per cell.<sup>63</sup> Although this kd is 10-fold higher than the kd determined in murine cells, this disparity may reflect technical limitations of the estimates of the G-CSF receptor dissociation constant. These studies were not performed under equilibrium conditions, and the association rate of G-CSF with its receptor in human neutrophils appeared to be slower than in murine cells, which would be reflected in a higher value for the apparent kinetic kd. The G-CSF receptors on human neutrophils were also "down-modulated" by exposure of cells to G-CSF, GM-CSF, bacterial lipopolysaccharide, and the chemotactic peptide f-met-leu-phe.<sup>63</sup> However, other studies have shown that G-CSF receptors are only minimally downregulated from the surface of human neutrophils by exposure to GM-CSF, while both TNF and TPA induced significant loss of G-CSF binding capacity that could be blocked by preincubation of cells with staurosporine (a potent inhibitor of protein kinase C) or protease inhibitors.<sup>64</sup> Exposure of human neutrophils to IL-6 or IL-2 has no demonstrable effect on G-CSF receptors.<sup>64</sup> The mechanism of receptor

down-modulation is unknown, but ligand- and TPA-induced receptor internalization is a commonly seen phenomenon in growth factor receptor biology, and has also been observed in the receptors for M-CSF, GM-CSF, and IL-3.<sup>22,65,66</sup>

The murine G-CSF receptor has been identified in the WEHI-3B D<sup>+</sup> cell line by chemical cross-linking and found to have an apparent molecular mass (M<sub>r</sub>) of 150,000 daltons which, after binding of G-CSF and prolonged incubation, appeared to be degraded into smaller species of M<sub>r</sub> 50,000 and 90,000.<sup>67</sup> In the murine myeloid leukemia cell line NFS-60, a similar cross-linking analysis suggests that the G-CSF receptor in these cells consists of a single polypeptide of M<sub>r</sub> in the range of 100,000 to 130,000 daltons with the variability probably due to differences in glycosylation status of the receptor.<sup>68</sup> The receptor has been solubilized from the NFS-60 cell line, and the purified receptor has been shown to bind G-CSF with two affinities. The first represents a high-affinity binding activity (kd = 120 to 360 pmol/L) that appears to represent an oligomeric form of several copies of the G-CSF receptor protein, while the second is a moderate-affinity binding activity (kd = 2.6 to 4.2 nmol/L) that is apparently a monomeric form of the solubilized G-CSF receptor protein.<sup>68</sup> Because intact NFS-60 cells bind G-CSF with high affinity, this suggests that the intact membrane-bound form of the G-CSF receptor could exist as an oligomer. These data are summarized in Table 2.

Receptors for G-CSF have also been demonstrated and studied on a variety of other cells, including human myeloid leukemia cells and leukemic cell lines,<sup>69-74</sup> human placenta and trophoblastic cells,<sup>75</sup> human vascular endothelial cells,<sup>76</sup> and cell lines derived from human small cell carcinoma of the lung.<sup>77</sup> Despite the presence of G-CSF binding activity on such cells, the functional significance of G-CSF receptors expressed on most nonhematopoietic cells remains unclear. In contrast, G-CSF has been shown to act as a potent proliferative stimulus for some cases of myeloid leukemia. Overall, significant variability has been reported in the presence and numbers of G-CSF receptors on human myeloid leukemia cells. The affinity of the G-CSF receptors, if present, on acute or chronic myeloid leukemia cells seems to be similar to the affinity of the receptor of normal neutrophils, with a kd of approximately 300 pmol/L.<sup>72-74</sup> Additionally, G-CSF receptors on fresh acute myeloid leukemia (AML) blasts are downregulated by TNF in a manner similar to G-CSF receptors on normal mature

**Table 2. Characteristics of the G-CSF Receptor**

Single chain polypeptide
Murine 812 amino acids
Human 812 or 759 amino acids
100-150-Kd molecular weight
Two binding affinities demonstrable
High affinity (kd = 65 to 360 pmol/L): oligomeric
Moderate affinity (kd = 2,600 to 4,200 pmol/L): monomeric
50-500 cell surface receptors per cell
Downregulated by prior exposure to G-CSF, TNF, TPA, LPS, f-met-leu-phe, GM-CSF, IL-3
No endogenous kinase domain

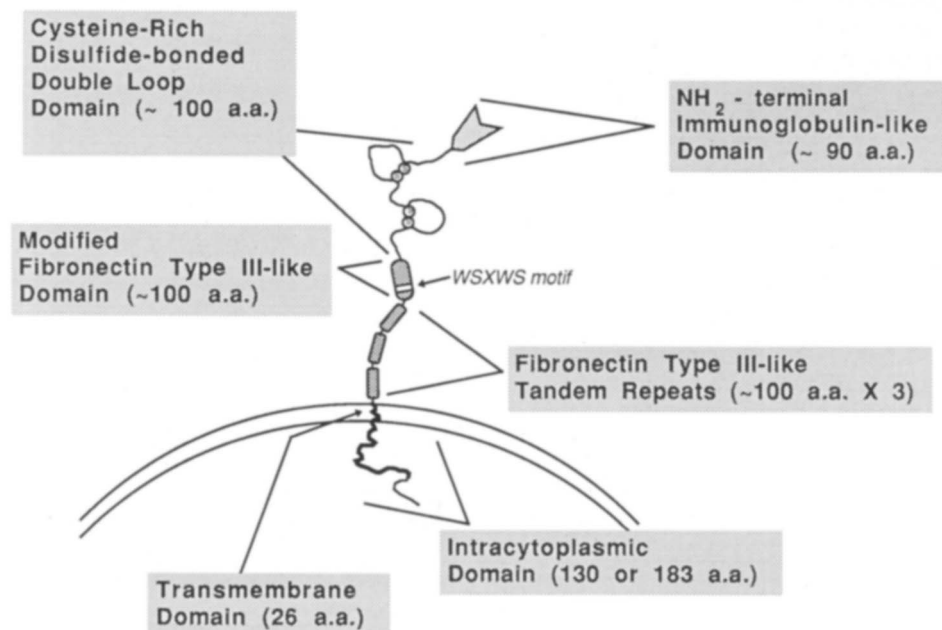


neutrophils.<sup>64</sup> However, a large subset of blast cells from patients with AML fail to express detectable G-CSF receptors, consistent with earlier observations that only about half of cases of AML proliferate in response to G-CSF in vitro.<sup>78,79</sup> No clear correlation has been shown between the number of G-CSF cell surface receptors expressed and the ability of the leukemic clonogenic cells to proliferate in vitro in response to exogenous G-CSF, suggesting that other aspects of the signal transduction pathway may be perturbed in these cells.<sup>69,71,72</sup>

As noted previously, the physiologic significance of G-CSF receptors on the surface of nonhematopoietic cells is unclear. These G-CSF receptors are similar to the receptors expressed on cells of the neutrophilic lineage, binding G-CSF with high affinity and present on the surface of these nonhematopoietic cell types in sparse copy numbers (< 500 receptors/cell).<sup>75-77</sup> G-CSF induces human vascular endothelial cells to proliferate as well as to migrate.<sup>76,80</sup> In vivo, G-CSF was shown to induce an angiogenic response when introduced into rabbit corneas via a slow-release pellet. This neovascularization induced by G-CSF was not as dramatic as that associated with basic fibroblast growth factor (bFGF); however, the combination of G-CSF plus bFGF appeared to produce more of an angiogenic response than either factor alone.<sup>80</sup> There remains some controversy about G-CSF receptors and action on vascular endothelial cells, since Yong et al<sup>81</sup> have reported that they have been unable to demonstrate high-affinity G-CSF receptors on these cells and that G-CSF had no functional effects on cultured endothelial cells.

The molecular cloning of cDNAs encoding the murine<sup>82</sup> and human<sup>83</sup> G-CSF receptors has been reported, in each case by using a strategy of expression cloning from cDNA libraries derived from NFS-60 murine myeloid leukemia cells or from human placenta, respectively. The putative murine G-CSF receptor consists of 812 amino acids with a

single transmembrane domain and a cytoplasmic domain of 187 amino acids.<sup>82</sup> Two human cDNAs have been cloned that encode predicted G-CSF receptors of 759 and 812 amino acids, the latter of which is more homologous to the murine G-CSF receptor<sup>83</sup> (Fig 2). These two human G-CSF receptors are identical except for different carboxyl terminus sequences; this difference appears to result from alternative processing of the same gene product.<sup>83</sup> The extracellular domain of the G-CSF receptor contains several structural motifs characteristic of other proteins. This mosaic structure, in which a composite is formed from small regions found in other molecules, is characteristic of the growing family of hematopoietic growth factor receptors, including the receptors for G-CSF, GM-CSF, erythropoietin, IL-3, IL-4, IL-6, IL-7, and the  $\beta$ -chain of the IL-2 receptor.<sup>84,85</sup> The shared structural features of this family include conservation of four cysteines, a W-S-X-W-S motif near the transmembrane domain, and the presence of several repeats of a sequence related to a fibronectin type III domain. Additionally, a significant portion of the extracellular domain of the G-CSF receptor exhibits a striking similarity to the prolactin receptor. Also, the structure of the extracellular domain of the G-CSF receptor exhibits some similarity to the NCAM family of adhesion molecules, possibly suggesting a role for the G-CSF receptor in adhesive interactions. The transmembrane domain and the intracytoplasmic portion of the G-CSF receptor are related to corresponding regions of the IL-4 receptor, with sequence homology of approximately 50%.<sup>82</sup> These structural similarities in the G-CSF and IL-4 receptors suggest that there may be similarities in the pathways of signal transduction triggered by these two receptor-ligand systems. The analysis of sequence of the G-CSF receptor does not suggest that the receptor itself functions as a kinase, and the mechanisms responsible for signal transduction through this receptor are not yet known.



**Fig 2.** Schematic representation of a cloned receptor for human G-CSF. The mosaic structure composed of distinct domains is characteristic of receptor molecules for several hematopoietic growth factors and other cytokines. Reproduced and adapted from the *Journal of Experimental Medicine*, 1990, volume 172, page 1567, by copyright permission of the Rockefeller University Press.<sup>83</sup>



Based on the lack of obvious effector domains for signal transduction within the cytoplasmic domain of the G-CSF receptor, and considering the homology of both G-CSF and the G-CSF receptor to IL-6 and the IL-6 receptor, respectively, it is possible that the G-CSF receptor may associate with another polypeptide to transduce appropriate signals. This system would then be analogous to the interaction of the gp130 molecule with the IL-6 receptor.<sup>27</sup> It is likely that many or all of the members of this receptor family are multimeric. Typically in these receptors, there are two proteins that interact directly with the ligand; either one of these proteins singly would bind ligand only with low affinity. Reconstitution of a high-affinity receptor would require coexpression of both binding proteins. In contrast to the prediction of this hypothesis, it is notable that expression of the single polypeptide chains of either the cloned murine or human G-CSF receptor results in high-affinity binding (kd of approximately 300 pmol/L), similar to binding activity of the native G-CSF receptor.<sup>82,83</sup>

The signal transduction mechanisms activated by the G-CSF receptor remain poorly understood. Binding of G-CSF to its receptor on human umbilical vein endothelial cells rapidly induces an increase in intracellular pH, which appears to be due to activation of a  $\text{Na}^+/\text{H}^+$  exchange mechanism.<sup>86</sup> In contrast, exposure of human neutrophils to G-CSF does not induce any evident acute effects on several indicators of signal transduction, including the intracellular concentration of free calcium ions, the resting transmembrane electrical potential, and intracellular pH.<sup>87</sup> An accelerated rate of membrane depolarization induced by f-met-leu-phe is noted in neutrophils after preincubation with G-CSF, without a corresponding effect on the rate of increase in intracellular free calcium.<sup>87</sup> G-CSF does appear to directly induce release of arachidonic acid from cell membrane phospholipids, suggesting the early activation of a phospholipase following exposure of mature neutrophils to G-CSF. It is unlikely that G-CSF acts directly through a protein kinase C-dependent mechanism because neutrophils exposed to G-CSF did not translocate protein kinase C from the cytoplasm to the cell membrane.<sup>87</sup>

After binding of G-CSF to NFS-60 murine leukemia cells, there is a rapid (within 15 minutes) increase in the affinity of binding of radiolabeled guanosine triphosphate to isolated cell membranes. This increased binding affinity can be blocked by pretreatment of the cell membranes with cholera toxin. Additionally, G-CSF binding induces a five-fold increase in the concentration of intracellular cyclic adenosine monophosphate (cAMP) which peaks within 15 minutes and then rapidly declines toward baseline.<sup>88</sup>

Many growth factor receptors activate protein tyrosine kinases.<sup>89</sup> This has been shown for several members of the hematopoietic growth factor receptor family, including IL-2, IL-3, and GM-CSF. G-CSF also activates a tyrosine kinase, but the substrates of this kinase, at least in neutrophils, are different than those associated with the GM-CSF receptor-associated tyrosine kinase. In particular, G-CSF stimulates rapid tyrosine phosphorylation of a protein of approximate M<sub>r</sub> 80,000 (Okuda K, Griffin, JD: in preparation). Until the identities of these proteins are determined,

the role of tyrosine kinases in the signal transduction pathways used by G-CSF and GM-CSF will be unclear.

#### BIOLOGIC ACTIVITIES OF G-CSF

*Action of G-CSF on normal hematopoietic progenitor cells in vitro.* The primary effects of G-CSF on normal hematopoietic cells are limited to cells of the neutrophil lineage. In vitro, G-CSF stimulates proliferation and differentiation of neutrophil colony-forming cells and alters several functions of mature neutrophils.<sup>90</sup> G-CSF acts on a relatively mature progenitor cell population that is primarily committed to neutrophilic differentiation. Cultures of human marrow progenitors enriched for a relatively primitive population expressing the CD34 antigen in the absence of CD33 expression ( $\text{CD34}^+/\text{CD33}^-$ ) show minimal response to G-CSF in terms of neutrophil colony formation. However, G-CSF can effectively stimulate colony formation from a somewhat more mature marrow progenitor cell population committed to myeloid differentiation ( $\text{CD34}^+/\text{CD33}^+$ ).<sup>91</sup>

The kinetics of G-CSF stimulation of progenitor cells has been studied.<sup>92</sup> Exposure of human marrow cells to a brief pulse of G-CSF, followed by washing and culture under serum-containing, non-CSF-supplemented conditions, induced a single round of cell division by mature progenitor cells (eg, promyelocytes and myelocytes). Culture of human marrow enriched for early blast cells showed that pulse exposure to G-CSF was unable to stimulate these very early progenitors to traverse the cell cycle.<sup>92</sup> In vitro, liquid culture of  $\text{CD34}^+$  marrow progenitor cells with G-CSF results in loss of the CD34 antigen within 6 days, suggesting that G-CSF alone does not support self-renewal of primitive hematopoietic cells.<sup>91</sup> Data from long-term bone marrow cultures (LTBMC) suggest that G-CSF does not alter the longevity of the active hematopoietic compartment in the LTBMC, indicating that there is no early exhaustion of more primitive progenitors due to G-CSF.<sup>93</sup>

The interactions of G-CSF with other hematopoietic growth factors in stimulating proliferation of progenitor cells are complex. Particularly in combination with other factors, G-CSF can stimulate the survival and early stages of proliferation of a broad range of progenitor cells in vitro. Thus, exposure of progenitor cells to the combination of G-CSF and GM-CSF results in the appearance of more numerous, larger colonies than with either factor alone.<sup>90,94</sup> This property may, in part, be responsible for the observation that G-CSF can stimulate monocytic colonies under certain culture conditions. In culture systems where other CSFs are supplied by "contaminating" accessory cells, the activities of G-CSF may extend to a multilineage stimulation of hematopoietic cells. As noted previously, G-CSF was initially described as a multilineage-stimulating "pluripoiectin," but, after depletion of accessory cells from culture conditions, no direct pluripotent stimulatory activity of G-CSF was noted.<sup>13</sup>

The apparent effects of G-CSF on primitive stem cells are of particular interest. G-CSF alone does not support the sustained growth of murine multipotential progenitor cells, but in cultures of spleen cells from 5-fluorouracil (5-Fu)-treated mice, blast cell colonies appeared significantly

earlier *in vitro* if both G-CSF and IL-3 were present, compared to cultures supplemented with either factor alone.<sup>95</sup> The total number of blast cell colonies was not increased by the addition of G-CSF to IL-3, but the rate of appearance of these colonies was augmented by G-CSF,<sup>95</sup> suggesting that this combination either (1) hastens entry into the cell cycle of a larger fraction of the resting primitive progenitor cell pool, or (2) accelerates transit through the cell cycle so that colonies appear earlier. However, after the blast colonies appear, their rate of growth is the same whether or not G-CSF is present. G-CSF also exhibits synergistic activity with IL-3 in promoting the development of more differentiated CFU-GEMM colonies from murine marrow cells harvested 2 days post 5-FU. In contrast to the data regarding the effects of G-CSF plus IL-3 on induction of blast cell colonies, CFU-GEMM colonies both (1) appear more rapidly and (2) are more numerous when these progenitor cells are cultured with both IL-3 plus G-CSF compared with cultures containing either cytokine individually.<sup>96</sup> G-CSF has also been shown to synergize with IL-3 in stimulation of colonies derived from early progenitor cells enriched from human peripheral blood, resulting in an increase in colony numbers (day 14 CFU-GM) compared with IL-3 alone; G-CSF by itself was completely inactive in supporting proliferation of these colonies.<sup>97</sup> Besides IL-3, G-CSF has been shown to modulate the biologic response of primitive, multipotent progenitor cells to other cytokines, including IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6, but not GM-CSF, M-CSF, erythropoietin, IL-2, IL-4, or IL-5.<sup>96</sup>

IL-4 appears to interact with G-CSF in a complex manner to enhance responsiveness of progenitor cells committed to the neutrophilic lineage. Several studies have shown that IL-4 can augment the proliferation of cells which form neutrophilic colonies in response to G-CSF,<sup>98-101</sup> although the combination of IL-4 plus G-CSF does not show any increased activity on multipotent progenitors compared with the individual factors.<sup>96</sup> It appears that IL-4 must be present early in the cultures to have any enhancing effect, suggesting that IL-4 may sensitize committed cells to the effects of G-CSF.<sup>101</sup> The structural relationships shared by the G-CSF receptor and the IL-4 receptor suggest that the molecular mechanisms responsible for this phenomenon may be of great interest.

G-CSF has also been shown to interact with IL-6 in stimulating colony formation from progenitor cells obtained from mice treated with 5-FU. No stimulation of colony growth was noted when murine marrow cells harvested 2 days following 5-FU administration were cultured with G-CSF, IL-6, or the combination of G-CSF plus IL-6. However, synergistic stimulation of colony growth was induced by the combination of IL-6 plus G-CSF when marrow cells harvested 4 days after 5-FU treatment were cultured,<sup>102</sup> suggesting that the interaction of G-CSF with IL-6 may occur at the level of a more mature progenitor cell (because the marrow harvested later will have a greater concentration of more mature progenitor cells). At low concentrations of G-CSF, IL-6 has been reported to inhibit G-CSF-induced colony formation by human bone marrow

cells; this effect does not occur when marrow cells are cultured in higher concentrations of G-CSF.<sup>103</sup>

Using mice myelosuppressed by 5-FU administration, Moore and Warren<sup>104</sup> have demonstrated synergistic activity of G-CSF with IL-1 $\alpha$  in stimulating multilineage hematologic recovery *in vivo*. Because of the obvious complexities of such an *in vivo* model system, it is not possible to determine whether this synergy is due to a direct effect of IL-1 $\alpha$ , or whether it is mediated by secondary cytokines produced in response to IL-1.

In summary, it can be concluded that G-CSF commonly exhibits synergistic hematopoietic activity with other cytokines *in vitro*. This synergy may be particularly striking when G-CSF is combined with other cytokines that act on more primitive progenitor cells, such as IL-3 or IL-6. Therefore, it is not surprising that data have been reported regarding the synergistic activity of G-CSF in combination with the cloned ligand for the *c-kit* proto-oncogene,<sup>105</sup> because the *c-kit* ligand has been shown to act on an extremely early cell population within the hematopoietic hierarchy.<sup>106,107</sup> It is important to note that many of the synergistic interactions between G-CSF and other cytokines have yet not been observed *in vivo*. For example, no *in vivo* synergy has been noted in cynomolgous monkeys treated with G-CSF in combination with either IL-4 or IL-6 (K. Welte, L. Souza: personal communication, June 1991). It is clear that there are many potentially important variables (such as dose and schedule of administration) that might obscure synergistic activities of combination cytokines *in vivo*. Further investigations in this field will clearly be necessary to define the biologic interactions between G-CSF and other hematopoietic cytokines *in vivo*.

*Preclinical activity of G-CSF in vivo.* Recombinant human (rh) G-CSF has been shown to stimulate granulopoiesis and release of neutrophils into the blood when administered to Golden Syrian hamsters either by continuous intravenous infusion or by subcutaneous injection.<sup>108</sup> A single subcutaneous injection of rhG-CSF resulted in an increase in circulating granulocytes within 2 hours; the granulocyte concentration peaked by 12 hours and remained elevated for the next 36 hours before decreasing slowly back to baseline.<sup>108</sup> These acute changes in peripheral granulocyte counts were associated with increases in bone marrow cellularity, the number of colony-forming progenitor cells, and the percentage of marrow S-phase cells, all of which appeared within 12 hours after a single subcutaneous injection of G-CSF. In this same model, G-CSF accelerated both the onset of leukocyte nadir and leukocyte recovery by 1 to 1.5 days in hamsters after myelosuppressive doses of 5-FU.<sup>108</sup> In a rat model of neonatal hematopoiesis, rhG-CSF administered for 7 days resulted in a small increase in the total number of marrow committed progenitor cells (CFU-GM) without changes in extramedullary (liver and spleen) CFU-GM.<sup>109</sup> In another study, administration of very high-dose rhG-CSF to mice over a 5-day period resulted in a decrease in bone marrow cellularity, a decrease in marrow colony-forming cells (CFCs), and an increase in spleen weight and splenic CFCs. Additionally, pulmonary infiltration by mature neutrophils

was observed without gross evidence of pulmonary dysfunction.<sup>110</sup> These data suggest that G-CSF functions to redistribute hematopoietic progenitor cells from the marrow compartment to extramedullary sites.

rhG-CSF also stimulates myelopoiesis and release of neutrophilic granulocytes in primates.<sup>111</sup> In addition to the dose-dependent neutrophilia induced by rhG-CSF, administration of higher doses of G-CSF (10 or 100  $\mu\text{g}/\text{kg}/\text{d}$ ) was associated with a small increase (1.5- to 2.5-fold) in the absolute lymphocyte counts, with no effects noted on the levels of eosinophils, monocytes, platelets, reticulocytes, or hemoglobin. After 28 days of G-CSF administration at 10  $\mu\text{g}/\text{kg}/\text{d}$ , foci of extramedullary hematopoiesis were evident in the spleen by pathologic examination.<sup>111</sup> No gross toxicities were evident from administration of rhG-CSF either to hamsters or to primates *in vivo*.<sup>108,111</sup>

Monkeys myelosuppressed by administration of cyclophosphamide (60 mg/kg daily  $\times$  2) were studied to evaluate the effects of G-CSF on leukocyte recovery after cytotoxic chemotherapy.<sup>111</sup> When G-CSF was administered to one monkey 72 hours after the last dose of cyclophosphamide, the peripheral blood leukocyte counts reached only a very mild nadir and rapidly recovered to normal and supranormal levels compared with a control animal. In another animal, twice-daily subcutaneous injections of G-CSF were administered for 6 days before, during, and for 20 days after cyclophosphamide chemotherapy (60 mg/kg daily  $\times$  2). Peripheral blood leukocyte counts in this monkey decreased rapidly, then recovered to supranormal levels significantly sooner than the recovery in the control animal. This one monkey represents the only published data regarding concomitant *in vivo* administration of chemotherapy with rhG-CSF.<sup>111</sup> The effects of concomitant administration of cytotoxic agents with G-CSF may merit further study.

The effects of G-CSF on radiation-induced myelosuppression have been studied by several groups.<sup>112-116</sup> Generally, these studies have reported acceleration of hematopoietic recovery and associated increases in survival of mice or dogs after lethal doses of radiation. However, after very intensive radiation exposure (doses  $\geq$  10.5 Gy) in mice, no survival advantage was noted in mice treated with rhG-CSF,<sup>114</sup> suggesting that there is a limiting level of radiation-induced damage beyond which rhG-CSF might not be effective. In transplants of major histocompatibility complex-matched marrow to lethally irradiated dogs, the median time to recovery of 1,000 neutrophils/ $\text{mm}^3$  was 8 days for dogs receiving adjunctive rhG-CSF, compared to 14 days in control transplanted dogs without rhG-CSF.<sup>116</sup> Administration of rhG-CSF to monkeys treated with supralethal total body irradiation followed by autologous bone marrow transplantation resulted in a decreased time to neutrophil recovery compared with control animals.<sup>117</sup> This acceleration of recovery appeared to be dose-dependent and was studied using very high doses of rhG-CSF (50 to 100  $\mu\text{g}/\text{kg}/\text{d}$ ).

The canine model of cyclic neutropenia (CN) in gray collie dogs has yielded important data regarding the effect of G-CSF on hematopoiesis. When rhG-CSF (5  $\mu\text{g}/\text{kg}$  twice daily by subcutaneous injection) was administered to ani-

mals with CN, the predicted periods of neutropenia did not occur, although the leukocyte counts still cycled at a new, higher level.<sup>118</sup> These dogs also exhibited an increase in circulating monocyte counts while rhG-CSF was administered. Interestingly, rhGM-CSF administered to CN dogs in this same dose and schedule did not significantly alter the cycling of the circulating neutrophil levels.<sup>118</sup> Other investigators have also observed that treatment of CN dogs with rhGM-CSF serves to increase the peaks of cycling neutrophil counts without blocking the nadir periods of neutropenia.<sup>119</sup>

In an effort to determine the effects of chronic exposure to aberrantly high circulating levels of G-CSF, murine G-CSF was inserted into a retroviral expression vector that was then used to infect murine marrow cells used to reconstitute lethally irradiated mice.<sup>120</sup> This model system resulted in a sustained neutrophilia evident in the peripheral blood as well as an infiltration of neutrophilic granulocytes in various organs, most notably the liver and lungs. The granulocytic hyperplasia did not represent a malignant disorder because no tumors developed in secondary animals transplanted with bone marrow or spleen cells from the primary transplant recipients. In contrast to the multi-system tissue damage associated with chronic overproduction of either GM-CSF<sup>121</sup> or IL-3,<sup>122,123</sup> no premature death or significant organ damage was noted in mice that overexpress G-CSF, even after periods as long as 6 months. In particular, no lung damage was noted despite the infiltration of pulmonary alveoli by excessive numbers of mature granulocytes.<sup>120</sup>

Administration of rhG-CSF to normal dogs for 30 days has been reported to result in the development of neutralizing antibodies against human G-CSF, associated temporally with a prolonged neutropenia in the animals.<sup>59</sup> These polyclonal anti-G-CSF neutralizing antibodies also blocked the hematopoietic activity of recombinant canine G-CSF *in vitro*, and plasma from a neutropenic post rhG-CSF dog induced neutropenia when infused into a normal dog.<sup>59</sup> These data suggest that blocking the action of endogenous G-CSF by immune mechanisms leads to a neutropenic state *in vivo* and are the first data which suggest that endogenous G-CSF may be absolutely necessary for the maintenance of normal neutrophil levels in the circulating blood *in vivo*. This model system should prove useful for further definition of the physiologic role played by G-CSF in normal hematopoiesis.

*Action of G-CSF on mature effector cells.* In addition to stimulating proliferation of the precursor cells of neutrophilic granulocytes, G-CSF also enhances the effector functions of mature neutrophils. Exposure of human neutrophils to G-CSF *in vitro* is not a sufficient stimulus for release of superoxide. However, a brief (5 to 10 minutes) preincubation of neutrophils with G-CSF "primes" the cells and enhances the superoxide production by these cells following exposure to the chemotactic stimulus f-met-leu-phe.<sup>124,125</sup> If neutrophils are first adhered to proteins immobilized on the surface of a culture dish, G-CSF alone is sufficient to induce a delayed respiratory burst, in contrast to nonadherent neutrophils.<sup>126</sup> This "priming" effect of



G-CSF on neutrophils is specific for responses triggered via receptor-mediated mechanisms, including f-met-leu-phe and wheat germ agglutinin, but is not seen for non-receptor-mediated neutrophil activating agents such as phorbol esters and the calcium ionophore ionomycin.<sup>127</sup> G-CSF has also been reported to increase surface expression of the CD11b antigen (the C3bi receptor) on neutrophils in vitro with a concomitant increase in adherence of neutrophils to nylon fibers.<sup>127</sup> Also, G-CSF dramatically upregulates the affinity for ligand of the neutrophil-endothelial cell homing receptor LAM-1<sup>128</sup> while not changing the level of LAM-1 expression on the surface of mature neutrophils.<sup>129</sup> These rapid changes in adhesion molecules may contribute to the acute, but transient, neutropenia observed in humans<sup>130</sup> and mice (Griffin JD, Koenigsman M, Demetri GD, Tedder TF: unpublished data, July 1991) that occurs briefly after administration of pharmacologic doses of rhG-CSF.

Exposure to G-CSF alone does not induce degranulation by mature human neutrophils.<sup>87</sup> In vivo, administration of pharmacologic doses of G-CSF has been associated with elevations in the circulating levels of leukocyte alkaline phosphatase, suggesting an alteration in secondary granule formation by relatively mature granulocytic progenitor cells.<sup>131,132</sup> The morphology of circulating neutrophils changes in response to G-CSF administration in vivo, with the induction of a moderate left shift in the neutrophil differential count and the appearance of mature neutrophils that exhibit prominent Dohle bodies and toxic granulations.<sup>131,133</sup> Neutrophils that exhibit the Pelger-Huet anomaly change their abnormal nuclear morphology in response to in vitro incubation with G-CSF, with an enhancement of nuclear segmentation evident within 24 hours.<sup>134</sup> This suggests that G-CSF may play a role in the developmental process of nuclear segmentation in maturing neutrophils.

G-CSF has been reported to enhance other effector cell functions, including stimulation of antibody-dependent cellular cytotoxicity (ADCC).<sup>12,135</sup> Induction of interferon- $\alpha$  gene expression and protein secretion has been reported after exposure of human neutrophils to G-CSF in vitro.<sup>136</sup> Because interferon- $\alpha$  has been reported to inhibit colony formation by granulocytic neutrophils, these investigators suggested that G-CSF-induced production of interferon- $\alpha$  might function as part of the regulatory mechanisms which control granulopoiesis by inhibiting neutrophil production in the presence of high levels of G-CSF.<sup>136</sup>

G-CSF can activate mature effector cell functions in neutrophilic granulocytes in vivo as well as in vitro. In primates, neutrophils obtained from animals treated in vivo with rhG-CSF demonstrated normal mobility in an in vitro chemotaxis assay but exhibited enhanced function by the nitroblue tetrazolium (NBT) reduction assay.<sup>111</sup> In a rat model of experimental neonatal sepsis, rhG-CSF administered prophylactically for 7 days resulted in a decreased mortality in response to group B streptococcal sepsis in combination with antibiotics compared with controls that did not receive rhG-CSF pretreatment.<sup>109</sup> Administration of rhG-CSF without subsequent antibiotics did not increase survival after experimental induction of streptococcal sepsis, although death was delayed somewhat compared with

controls. Treatment with rhG-CSF and antibiotics after experimental induction of streptococcal sepsis in neonatal rats without the presepsis "prophylaxis" period of administration failed to enhance survival compared with antibiotic therapy alone.<sup>109</sup> Limited data also suggest that treatment with G-CSF may augment resistance to *Pseudomonas* sepsis in murine models of immunocompromised states from either cytotoxic chemotherapy or after burn injury.<sup>137,138</sup>

In summary, as with several other hematopoietic growth factors such as GM-CSF and M-CSF, G-CSF exhibits the ability to enhance functional activities of the mature cells whose proliferation it stimulates, specifically the neutrophilic granulocytes.

*Action of G-CSF on hematopoietic progenitor cells in hematologic disorders.* Because G-CSF was identified on the basis of its ability to differentiate murine leukemia cells, the effects of G-CSF on human leukemic cells are of considerable interest. G-CSF exerts a potent proliferative stimulus for the clonogenic cells in a subset of cases of human acute myeloblastic leukemia.<sup>78,79,139,140</sup> This proliferative effect of G-CSF is evident in both short-term colony cultures and long-term cell suspension cultures.<sup>78</sup> In the initial characterization of recombinant human G-CSF, there was a suggestion that G-CSF might possess the ability to induce the differentiation of fresh human leukemia cells in vitro.<sup>14</sup> However, evaluation of a larger number of human leukemia specimens has not shown a strong differentiative activity of G-CSF, at least in vitro.<sup>78,79</sup> In a model system studying growth of a radiation-induced murine leukemia cell line, G-CSF was shown to induce proliferation of the leukemic cells in vitro. However, when leukemic cells were inoculated into syngeneic mice, administration of G-CSF did not accelerate overt development of leukemia. In fact, G-CSF administration was associated with an increase in the mean survival time compared with controls and a delay in the appearance of leukemic cells in the circulating peripheral blood compartment.<sup>141</sup> Therefore, it is possible that the clinical effects of exogenous G-CSF administered to patients in vivo may not be entirely predictable on the basis of in vitro analyses of leukemic clonogenic cell proliferation. In conjunction with the data from murine cell line model systems, these data support the investigation of exogenous G-CSF in the treatment of patients with leukemia treated under research protocols designed to evaluate the clinical effects of this agent.<sup>142</sup>

Human leukemia cells in culture exhibit heterogeneous responses to G-CSF in combination with other cytokines. In one study, the majority of leukemia cases examined by in vitro colony assays demonstrated a synergistic stimulation of clonogenic cell colony formation by the combination of G-CSF plus GM-CSF.<sup>143</sup> In a fraction of cases, IL-4 has been shown to augment G-CSF-induced proliferation of AML cells and enhance colony formation by clonogenic cells.<sup>100</sup> In studies of human leukemia cell lines, G-CSF has variable effects in combination with other cytokines: both synergistic activity in enhancing proliferation and antagonism with an arrest of proliferation were noted in different cell lines exposed to the combination of G-CSF plus GM-CSF.<sup>144</sup> This complexity of growth responses to hemato-



poietic cytokines by leukemia cells reflects the variability inherent in the biology of human AML.

The expression of hematopoietic growth factor genes and the production of hematopoietic cytokines by leukemia cells has been shown in human AML *in vitro*.<sup>140,145,146</sup> In addition to expression of the GM-CSF gene and production of that cytokine, another subset of human leukemias has also been shown to express the gene for G-CSF and to produce measurable amounts of this factor.<sup>140,147</sup> No correlation has been evident between G-CSF gene expression and the ability of AML cells to grow in culture autonomously without supplementation by an exogenous source of hematopoietic growth factors.<sup>140</sup> Thus, the significance of these *in vitro* observations in the pathophysiology of human leukemia remains uncertain.

The effects of hematopoietic growth factors on progenitor cells from patients with cyclic neutropenia have been studied in detail.<sup>148,149</sup> The concentration of G-CSF required to stimulate colony formation in cultured bone marrow mononuclear cells from patients with cyclic neutropenia is increased compared with controls; this 5- to 10-fold decrease in G-CSF responsiveness may play a role in the pathophysiology of this clinical syndrome and in the clinical therapeutic effects of G-CSF in this disorder.<sup>150</sup>

Bone marrow progenitor cells from patients with myelodysplastic syndromes (MDS) have been studied to evaluate the proliferative versus the differentiative signals induced by G-CSF as well as other cytokines.<sup>151,152</sup> Stimulation of clonogenic cell proliferation from MDS marrow mononuclear cells was seen with G-CSF, but this effect was much less than the stimulation induced by GM-CSF. Conversely, G-CSF appeared to induce evidence of granulocytic differentiation in MDS marrow cells more readily than GM-CSF. This differentiative effect of G-CSF was most notable in cells obtained from patients with normal cytogenetics compared to those with karyotypic abnormalities. Both the proliferative and differentiative activities of G-CSF were diminished in MDS marrow cells compared with marrow obtained from normal human volunteers, suggesting that a partial block to growth factor responsiveness may be a fundamental biologic characteristic of this heterogeneous group of disorders.<sup>152</sup>

Two patients with Kostmann-type congenital neutropenia were studied for responsiveness to G-CSF.<sup>153</sup> These progenitor cells exhibited poor colony formation *in vitro* in response to either G-CSF or IL-3 alone, compared with normal controls. However, the combination of G-CSF with IL-3 led to increased colony formation when these factors were present simultaneously or when G-CSF exposure followed a preincubation of cells with IL-3. IL-3 did not stimulate colony formation from progenitor cells preincubated with G-CSF, consistent with the concept of the hierarchical activities of these hematopoietic growth factors.

*Action of G-CSF on nonhematopoietic cells.* As noted previously, receptors for G-CSF have been found on the surface of several nonhematopoietic cell types, including human placenta and trophoblastic cells,<sup>75</sup> human vascular endothelial cells,<sup>76</sup> and cell lines derived from human small

cell carcinoma of the lung.<sup>77</sup> No functional analyses have been performed on the placental or trophoblastic cells, and it is not clear if the G-CSF receptor expressed by these cell types is functional.<sup>75</sup> The work of Bussolino et al<sup>76,80</sup> has shown that G-CSF can stimulate both migration and proliferation of human vascular endothelial cells in culture, and that GM-CSF also shares these activities on endothelial cells.<sup>76,80</sup> However, G-CSF possessed minimal activity on cultured endothelial cells in a separate study.<sup>81</sup> Two human colon adenocarcinoma cell lines, HTB-38 and CCL 187, exhibit increased colony formation in a modified soft agar clonogenic cell assay when cultured in the presence of G-CSF.<sup>154</sup> Two human small cell lung carcinoma cell lines, H69 and H128, also showed an enhancement of colony formation when G-CSF was present in range of 10 to 100 pmol/L, similar to the concentrations required for half-maximal induction of colony growth from normal human bone marrow.<sup>77</sup> Overall, these data support the hypothesis that G-CSF can affect the growth and activity of certain types of nonhematopoietic cells. Generally, demonstration of these effects in nonhematopoietic cells except endothelial cells has required specialized culture assays, such as clonogenic assays in soft agar, rather than more standard proliferation assays such as <sup>3</sup>H-thymidine incorporation. This may suggest that these proliferative stimuli are relatively weak *in vitro*, and the significance of G-CSF stimulation of nonhematopoietic cells *in vivo* remains unclear. Nevertheless, the existence of responsiveness of G-CSF of any magnitude by nonhematopoietic cells may have important clinical consequences, particularly with long-term administration.

*Clinical trials with G-CSF.* The clinical investigation of the activities of G-CSF *in vivo* has been reviewed and will not be covered in depth here.<sup>155-160</sup> In brief, G-CSF was first evaluated in phase I clinical trials in cancer patients and was shown to be a potent, well-tolerated agent that could increase the numbers of circulating neutrophils in a dose-dependent manner.<sup>130-132</sup> No maximal tolerated dose has been determined for G-CSF because phase I trials of G-CSF have never shown any dose-limiting toxicities. Dose escalations of G-CSF within phase I studies have generally been stopped simply because of extreme, but largely asymptomatic, elevations in the numbers of mature neutrophils in the peripheral blood. In trials of G-CSF with combination chemotherapy for cancer, the adjunctive use of G-CSF diminished the depth and duration of the neutrophil nadir period. A randomized, double-blinded, placebo-controlled, phase III study of adjunctive G-CSF with standard-dose cyclophosphamide/doxorubicin/etoposide chemotherapy in patients with small cell carcinoma of the lung has recently been completed.<sup>161,162</sup> In this study, administration of G-CSF after cytotoxic chemotherapy was associated with significant reductions in the duration of severe neutropenia, the incidence of fever with neutropenia, the requirement for antibiotics, and the incidence of hospitalizations.<sup>161,162</sup> Due to the crossover design of this study, which allowed the use of open-label G-CSF to all patients following the occurrence of an episode of fever with neutropenia, very few patients remained on blinded placebo treatment for the

entire planned duration of the treatment protocol. Therefore, this study does not definitively answer questions regarding whether the tumor response to chemotherapy or survival might be affected by the adjunctive use of G-CSF. Nevertheless, it was primarily on the strengths of this large-scale clinical study, in addition to numerous smaller clinical studies, that G-CSF was recommended for approval in December 1990 by an advisory panel of the United States Food and Drug Administration (FDA) for commercial use in cancer chemotherapy. This has led to the final approval of this molecule by the FDA in February 1991 for the remarkably broad indication of use with myelosuppressive chemotherapy for nonmyeloid malignancies, even before the final results of the phase III clinical trial were published.

G-CSF administration has been reported to increase the numbers of hematopoietic progenitor cells in the circulating blood by approximately 10-fold.<sup>131,163,164</sup> This increase in the concentration of peripheral blood progenitor cells associated with G-CSF treatment in vivo appears to be comparable in magnitude with that induced by administration of GM-CSF.<sup>165</sup>

G-CSF has been studied in association with high-dose chemotherapy and bone marrow transplantation. Adjunctive administration of G-CSF after autologous bone marrow transplantation appears to accelerate hematopoietic recovery.<sup>164</sup> The clinical use of G-CSF has also been explored in several other disease states, such as the myelos-

dysplastic syndromes,<sup>166,167</sup> congenital neutropenic states,<sup>168</sup> idiopathic neutropenia,<sup>169</sup> neutropenia associated with hairy-cell leukemia,<sup>170</sup> and cyclic neutropenia.<sup>150</sup> In each of these cases, G-CSF has been shown to exhibit significant activity in raising the circulating neutrophil counts. In myelodysplastic syndromes, a multicenter phase III clinical trial is currently underway to further define the clinical activity of G-CSF in these diseases. Specifically, it will be important to determine that administration of G-CSF to these patients is not associated with an increased rate of leukemic conversion, because in vitro data indicate that a significant fraction of leukemic blasts clearly has the capacity to respond to G-CSF with proliferation rather than differentiation. Additionally, it will be critical to determine whether the favorable laboratory results induced by G-CSF treatment of these disorders (eg, the increased numbers of circulating neutrophils, or the enhancement of neutrophil functions in vitro) result in beneficial clinical endpoints, such as a decreased incidence of infections.

In summary, despite the widespread availability of G-CSF for clinical use, many unanswered questions remain about the optimal clinical use of this potent agent. The knowledge gained from further investigations of the basic biology of G-CSF and from future clinical trials with this factor, both as a single agent and in combination with other hematopoietic growth factors, will be critical to determine rationally the role for this factor in patients with hematopoietic failure states or malignancy.

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