Human hematopoietic growth factors

Amy H Matayoshi*
Jeffrey M Nakamura MD** *(posthumously)*

The proliferation and the differentiation of bone marrow progenitor cells are regulated by glycosylated hematopoietic hormones. The major site of hematopoiesis is in the stromal matrix of the bone marrow, with secondary sites in the spleen, kidney and thymus. The cellular components of the blood are thought to be derived from a pluripotent stem cell. The peripheral blood cells possess limited life spans in the circulatory system. Their numbers must be maintained under normal conditions, and they must respond to the various environmental stresses placed upon the body. For example, the number of circulating leukocytes increases rapidly when an individual experiences severe infection[1,2].

Four of the major human myeloid growth factors will be discussed. Erythropoietin stimulates the production of red blood cells. Three colony-stimulating factors: Granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin III (IL-3), all influence the production and the maturation of the various types of leukocytes.

These hormones have been biosynthetically isolated and cloned, by means of recombinant DNA technology. The production of these factors in large quantities enables researchers to study their specific activities in great detail and to analyze their therapeutic relevance. Clinical trials are currently being conducted to further elucidate their potential for increasing the numbers and the activities of the various blood cells.

The hematopoietins

The hematopoietic proteins were first identified and purified from a variety of sources. In the late 1960s, the colony-stimulating factors were identified using cell-culture colony-formation assays. These assays consisted of 2 layers placed in a small laboratory dish. The first layer on the bottom of the dish, the "feeder layer," was comprised of various types of white blood cells in a semisolid medium. Bone marrow cells were placed on top of this feeder layer. When incubated, colonies of differentiated white blood cells formed the second layer. Altering the contents of the feeder layer resulted in the formation of various types and numbers of colonies[4].

The hematopoietic proteins were initially purified. In general, their amino-acid sequence was used to construct cDNA probes. These probes were used to isolate the desired gene from the human DNA library. The recombinant DNA was cloned and inserted into mammalian or bacterial vectors, along with various enhancers or promoters designed to amplify the expression of the gene for the large scale production of the hormone. The successful utilization of recombinant DNA technology allowed for extensive experimentation with these factors in clinical trials.

Erythropoietin was first isolated and purified from the urine of a patient with aplastic anemia[3]. The amino acid sequence was obtained and used to construct a DNA probe. This probe was used to identify the corresponding cDNA, which was then placed into mammalian cells. Mammalian

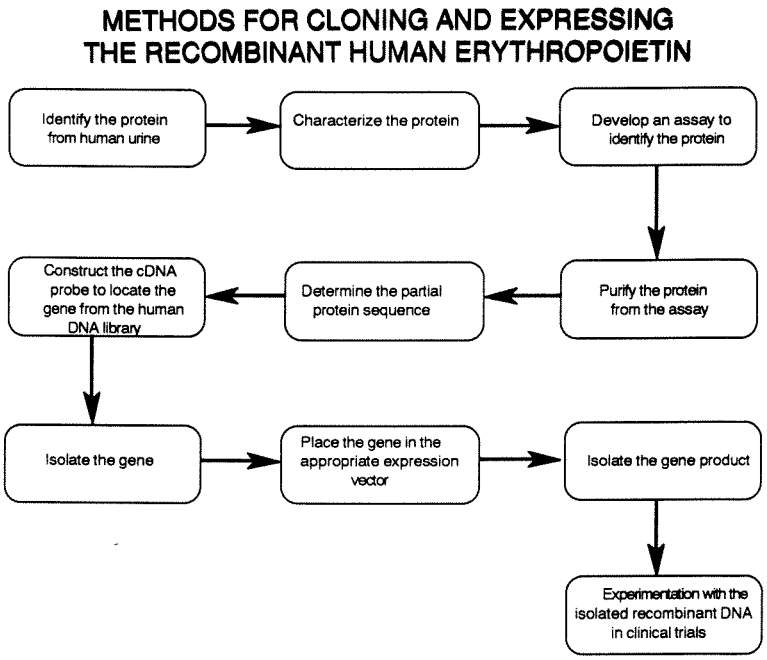

FIGURE 1: This figure outlines the steps utilized to clone and to express the gene for erythropoietin. Erythropoietin was the first of the hematopoietic growth factors to be manufactured biosynthetically. The form of erythropoietin used in clinical trials is produced only in mammalian cells.

(Continued on page 128) ➤

* Student
University of Redlands 1986
Redlands, California
** Associate Professor of Medicine, Division of Hematology
John A Burns School of Medicine
Director of Medical Education
Kuakini Medical Center
Honolulu, Hawaii

Received for publication April 23, 1991.

Innovation means never having to say, "It can't be done."

When you lease office equipment from GECC, you get more than hardware. You get innovative solutions.

Just call GECC, tell us your needs, and we'll help you decide whether leasing or buying is better for you. Yes, we can be objective. We offer loans for the purchase of office equipment, too.

Either way, you can count on innovative solutions from GECC, because there's nobody better in the business.

GECC Financial

EQUAL HOUSING LENDER

Member FDIC. GECC Hawaii Leasing is a division of GECC Financial—
a unit of General Electric Capital Corporation. 700 Bishop Street, 9th Floor: 527-8333.

(Continued from page 126)

cells were utilized as the host cell for erythropoietin because of the inability of bacterial and yeast cells to perform the necessary glycosylation steps in producing the biologically active form of the recombinant gene. The biosynthetically produced erythropoietin used in clinical trials is identical to the original, purified, urinary form.

The cDNA for G-CSF was developed using a structural approach. The natural hematopoietin was first purified from human cell lines and from normal placentae[1]. These purified factors were subjected to structural analysis and the resultant amino-acid sequence was used to construct the nucleotide sequence of different regions of DNA. Hybridization probes were constructed to select the G-CSF cDNA from the human DNA library to isolate the gene[2]. The isolated cDNA sequence was inserted into *E.coli* and also into mammalian cells to produce the functional hormone. The nonglycosylated form of G-CSF is used in clinical trials.

The trace physiological quantities of GM-CSF present in its natural form made purification difficult. Therefore, a different strategy was employed for the identification and the production of GM-CSF. HTLV-II transformed T-lymphoblast cell lines induced the production of GM-CSF and other regulatory hormones. The GM-CSF was partially purified. However, the limited yield of the natural protein made sequencing of the protein difficult. Therefore cDNA clones were transiently expressed in COS-1 (monkey) cells to construct cDNA libraries of approximately 300 recombinants[14]. These libraries were tested to see if they induced the secretion of GM-CSF in various assays.

The expression of GM-CSF mRNA in various cells such as lectin-stimulated peripheral blood lymphocytes, and some mature T-cell lines led to the further isolation of GM-CSF clones from the cDNA libraries[14]. The natural GM-CSF was purified, using column chromatography and 2 reversed-phase high-performance liquid chromatography (HPLC) steps. The recombinant GM-CSF was produced in large quantities using DNA transfection. Introducing the recombinant DNA into COS-1 cells allowed for production of recombinant GM-CSF, which also aided in the purification process. The recombinant protein showed no difference in specific activity as compared with the natural protein. The large amounts of the recombinant protein available facilitated the sequencing of the gene. Commercially, GM-CSF is produced in 3 different expression vectors: Mammalian cells, yeast cells, and bacterial cells *(E.coli)*[7]. These 3 vectors produce different levels of glycosylation as compared to the natural protein.

The cDNA for IL-3 was made through methods similar to those used to produce GM-CSF. IL-3 was the most difficult of the human growth factors to be isolated[2]. The IL-3 was purified in activated T-cells. The cDNA of a gibbon T-cell line encoding a multilineage CSF believed to be similar to IL-3 was used as a hybridization probe to identify the human gene[2,3]. The direct functional analysis of the products of cDNA clones introduced into mammalian cells in the appropriate expression vector was a novel approach to identify and to proliferate the hematopoietin.

Signal transduction

The mechanisms by which the hematopoietic growth factors affect their target cell have not yet been clearly delineated. Each growth factor binds to one specific receptor on the target cell surface[8,12]. The method and the prevalence of binding suggests that the individual receptors may affect the shape and, therefore, the ability of other receptors to bind to their specific factor. The synergistic effects of the various growth factors involve the interactions between the receptors and the growth factors bound to them[8]. The receptors may be activated transiently and may activate other growth proteins or secondary messengers such as cAMP or cGMP, depending on the mechanism of signal transduction in the stimulatory pathway[6,9]. The control of hematopoiesis lies in the proper identification of the cell surface receptors and their relationships to the other receptors during hematopoietic differentiation. In order to maintain normal growth as well as to promote rapid responses to environmental stresses, the cascading events which initiate the proliferation and the differentiation of the pluripotent stem cell must be identified. The make-up of the receptors for erythropoietin, IL-3, GM-CSF, and G-CSF have not yet been constructed. Because of the synergistic effects of the hematopoietic hormones, it has been difficult to isolate or to identify the receptors. The receptor structures are expected to be as diverse as the structures of the hormones themselves[9].

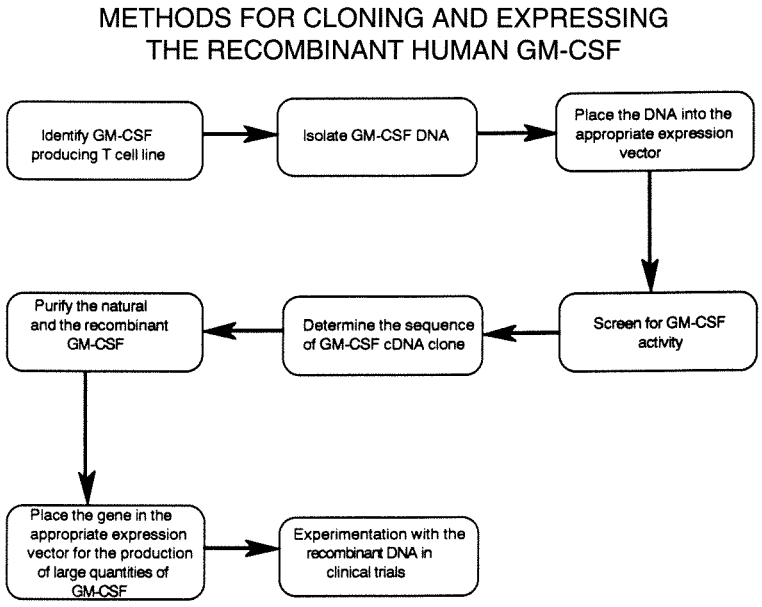

FIGURE 2: This figure outlines the steps utilized to clone and to express the gene for GM-CSF. IL-3 was cloned and expressed using similar methods. The trace quantities of these hormones caused difficulty in the large scale production of the genes. However, recombinant DNA technology has made it possible to yield large quantities of these hormones. This allowed for research into the clinical applications of these two hematopoietic hormones.

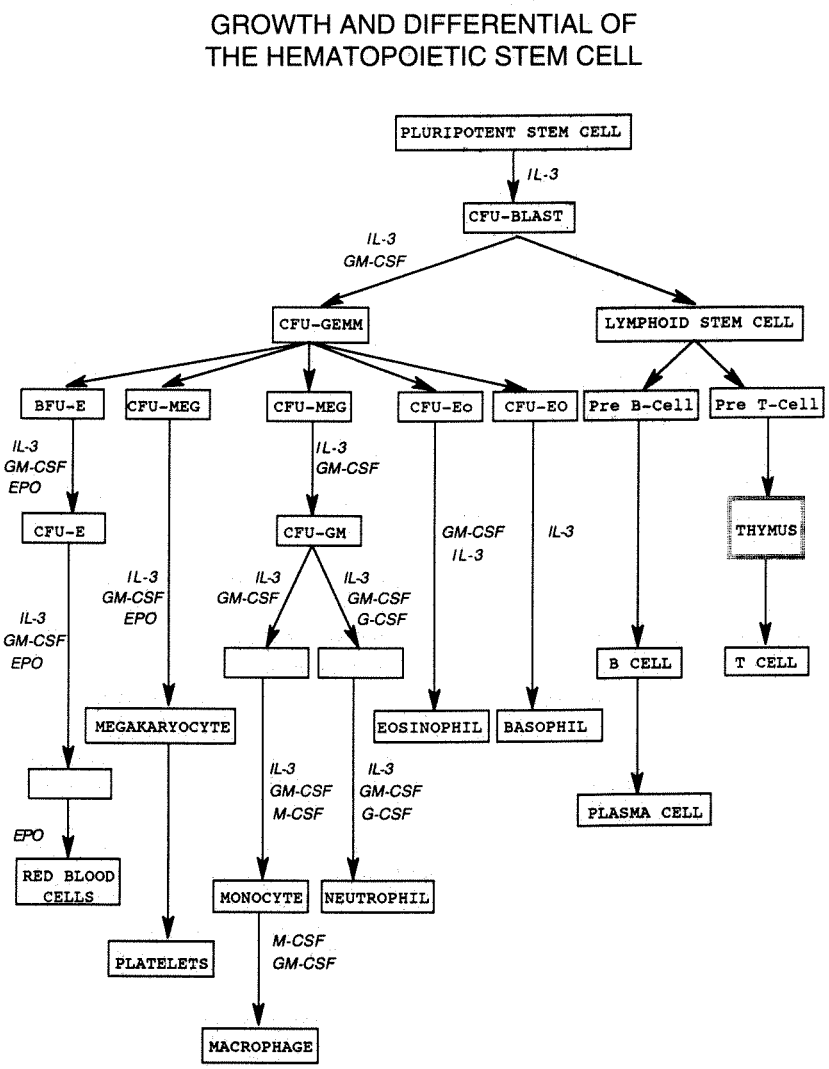

FIGURE 3: This figure provides an overview of hematopoiesis. The primary uncommitted progenitor cell is the pluripotent stem cell. A cell closely related to this cell is the CFU-BLAST, a multipotent cell identified using the CFU-blast colony assay[7]. The first step in the differentiation of the stem cell is a division into two major cell lineages. The growth of the lymphoid cells and the myeloid cells (CFU-GEMM) are influenced by the various hematopoietic hormones. The various progenitor cells were identified in vitro as CFU-GEMM (colony-forming unit, granulocyte-erythrocyte-monocyte-megakaryocyte); BFU-E (burst-forming unit-erythroid); CFU-MEG (CFU-megakaryocyte); CFU-Eo (CFU-eosinophil); CFU-E (CFU-erythroid); and CFU-GM (CFU-granulocyte-monocyte).

Erythropoietin

Erythropoietin regulates the production of red blood cells. It is produced in the peritubular cells of the kidneys, circulated via the plasma and excreted in the urine. Renal production of erythropoietin is directly proportional to hypoxemia. Erythropoietin was the first hematopoietic growth factor to be identified and cloned in mammalian cells. The gene for erythropoietin is located on the long arm of chromosome 7 (Table-1)[1,3,11].

The recombinant hormone used in clinical trials is derived from mammalian cells, due to the patterns of glycosylation necessary for the activation of the protein. The hormone was first used in patients with renal failure, a condition associated with a decrease in erythropoietin production and marrow sensitivity. Anemia associated with a decrease in the number of red blood cells often accompanies dialysis, and this is a result of the low levels of erythropoietin and the increase of inhibitors and toxins. Recombinant erythropoietin administration has been shown to increase the erythroid lineage, stimulating a dose-dependent rise in hematocrit levels. This has led to subjective improvement in sleeping and waking patterns, exercise capability, energy, appetite, and libido[1]. The side effects of erythropoietin were controlled by regulating the rise of hematocrit levels to 6% per month[1]. Hypertension may occur as a result of the increase in blood volume or in blood viscosity and must be closely monitored.

Anemias associated with decreased levels of erythropoietin are expected to benefit from erythropoietin therapy. However, reports have shown that anemias associated with normal or high serum erythropoietin levels also show improvement in response to erythropoietin therapy[1]. Patients with severe anemia or rheumatoid arthritis, for whom transfusions are contraindicated, may benefit from erythropoietin treatments[1].

Anemias accompanying various forms of therapy can also be treated with synthetic erythropoietin. For example, zidovudine (AZT) treatment in AIDS patients causes anemia[1]. Simultaneous administration of erythropoietin has been successful in combating the anemia in patients receiving AZT unless the serum erythropoietin levels were greater than 500 mu per milliliter before therapy[1]. This would allow for adequate dosage and duration of zidovudine treatment in patients with AIDS.

Erythropoietin also may be used on surgical patients, reducing the need for transfusion. Theoretically, the increase of erythrocyte production by erythropoietin will improve the patient's ability to recover from red-cell depletion. Erythropoietin would need to be administered before, during and after surgery. This would reduce the risk inherent in giving transfusions and perhaps ultimately the risk of the surgical procedure itself[4].

IL-3

IL-3 stimulates the proliferation of stem cells and committed progenitors of multiple lineage cells. It is an early-acting factor that stimulates proliferation of an enormous range of cell types. Clinical trials with the administration of IL-3 have begun but the results of these trials have not yet been reported. IL-3 was first produced by Yang et al in cultured monkey cells[7]. The inducible cellular source of IL-3 is the T-lymphocyte. The gene for IL-3 is located on the long arm of chromo-

(Continued on page 131) ➤

some 5[4,12,14]. The association between IL-3 and the other hematopoietic growth hormones located on this chromosome has not yet been determined.

GM-CSF

The first human GM-CSF cDNA was cloned and expressed in mammalian cells by Wong et al[4,7]. Along with IL-3, GM-CSF (a multipoietin) will target pluripotent cells and immature progenitor cells. It is not lineage specific, and it is required to be present throughout the process of differentiation. The gene for GM-CSF is also located on chromosome 5[4,12]. GM-CSF stimulates the growth of both granulocytic and monocytic colonies in a semisolid culture. GM-CSF has an early effect on myeloid development, and together with erythropoietin (late acting promoter of erythroid development), induces the formation of colonies containing erythroid and megakaryocytic cells.

GM-CSF also enhances the function of mature macrophages, eosinophils, and neutrophils. In various cells, it can cause increases in tumoricidal and phagocytic activity, intracellular killing, ADCC, superoxide production, immunoglobulin-mediated (opsonized) phagocytosis and chemotactic responsiveness (Handman and Burgess 1979; Lopez et al 1983, 1986; Vadas et al 1983; Weisbart et al 1986; Williamson and Brown 1987)[7]. GM-CSF also induces an increase in granulocyte aggregation in vitro (Arnaout et al 1986; Lopez et al 1986).

GM-CSF administration reduces the myelotoxic side-effects of chemotherapy for sarcoma. GM-CSF increases the neutrophil count; this can shorten the interval between cycles of chemotherapy. However, the ability to complete the cycle is not known to improve the survival rate or the response rate of the patient[1].

In bone marrow transplants, both mammalian recombinant GM-CSF and recombinant GM-CSF produced from yeast were tested. Mammalian cell GM-CSF did not significantly reduce the number of days that the leukocyte count was below 1,000/cubic millimeter. However, on the 14th day after treatment, a higher leukocyte count showed up and the incidence of bacteremia was reduced. Yeast-produced GM-CSF, in contrast, showed a shorter period of neutropenia (14 days as compared with 25 days in the control group); a decrease in the number of febrile days (6 days compared with 12 days); a decrease in the number of platelet units transfused (67 days compared with 84 days); and a reduction in the length of hospital stay (29 days compared with 41 days)[1]. There was also a reduced period of pancytopenia and a reduction in the infectious complications associated with autologous bone marrow transplants. However, it was not established whether there was also a decrease in the mortality rate of GM-CSF treated patients[1,7].

Aplastic anemia is caused by a decrease in the number of marrow stem-cells the result of an altered marrow microenvironment or immunologic disorder. GM-CSF improved the leukocyte count and bone marrow cellularity but with no increase in neutrophils. Therefore, the early progenitor cells affected may also require treatment with additional growth factors to be effective. However, these tests have not yet been conducted[1,7].

Continuous intravenous infusion with GM-CSF for 14

(Continued) ➤

PROPERTIES OF HUMAN COLONY-STIMULATING FACTORS

FACTOR	MOLECULAR WEIGHT (kd)	CHROMOSOME LOCATION	CELL PRODUCER	CELL STIMULATED	CLINICAL APPLICATIONS
EPO	34-39	7q11-q22	Peritubular cells of the of the kidney, Kupffer cells	late BFU-E, CFU-E, CFU-Meg	renal failure, anemias, preoperative & postoperative patients, AIDS
G-CSF	18-22	17q11.2-21	Monocytes, bone marrow stromal cells	CFU-G, HL60 cell line	bone marrow transplants, chemotherapy, neutropenias
GM-CSF	14-30	5q23-31	T lymphocytes, endothelial cells, bone marrow stromal cells, fibroblasts	CFU-GM, BFU-E, CFU-MIX, CFU-EO, CFU-Meg, Granulocytes, Monocytes, Eosinophils, KGI &HL60 cell lines	bone marrow transplants, chemotherapy, myelodysplasia, anemias, neutropenias, AIDS
IL-3	47-90	5q23-31	T lymphocytes	CFU-MIX, BFU-E, CFU-GM/G/M, CFU-EO, CFU-Meg	unknown

TABLE 1: This table lists the various properties of 4 hematopoietic hormones, erythropoietin, G-CSF, GM-CSF, and IL-3.

days in patients with pancytopenia due to myelodysplasia has demonstrated an increase in the total number of leukocytes (5 to 70 fold), granulocytes (5 to 313 fold), eosinophils, monocytes, and also a small increase in lymphocyte count. Maintenance therapy showed some multilineage effects. There was an increase in platelet and reticulocyte count, and an increase in hemoglobin concentration which caused a decrease in the necessity to transfuse red cell and platelets. However, the increase in platelet and erythrocyte count frequently did not occur; this was of minimal clinical importance[1,7].

The most significant characteristic of AIDS in patients is the decrease in T4 lymphocytes. The low levels and decreased functional abilities of neutrophils and monocytes in AIDS patients can be improved by the various treatments against the virus. However, Zidovudine (AZT) treatment has been shown to cause myelosuppression. In addition, antiretroviral, antimicrobial, or antitumor therapy used as therapy in AIDS patients can give rise to low blood counts. The use of GM-CSF and G-CSF have resulted in an increase in neutrophils, bands, eosinophils, and a slight increase in monocytes. The dose-dependent response to GM-CSF and G-CSF could benefit the patient's immune response and improve the hematologic tolerance to other drugs. These growth factors have also proven to be effective for long-term therapy, as shown by the absence of tachyphylaxis when given subcutaneously.

GM-CSF alone does modulate HIV activity. Studies indicate that there is an increase in HIV p24 antigen (viral specific protein) levels when patients are treated with GM-CSF alone. Therefore, the advantage of the administration of GM-CSF is being studied in conjunction with treatment with Zidovudine. Although GM-CSF counteracts the negative side-effects of some treatments, it is not yet clear if this also leads to an improved outcome for the patient with AIDS[1,7].

G-CSF

G-CSF is a late-acting factor that stimulates the growth of neutrophil colonies in vitro. G-CSF was first purified from human cell lines and placenta[1]. This factor was isolated and cloned by Souza et al in *E.coli*[4,7]. The inducible cellular source of G-CSF are the monocytes and bone marrow stromal cells. The gene for G-CSF has been located on the long arm of chromosome 17[4,12]. G-CSF also has a maturation-inducing effect and will act on mature effector cells to enhance their function. It acts only on the neutrophil colonies to increase antibody-dependent cell-mediated cytotoxicity (ADCC) and to enhance their phagocytic capability.

The nonglycosylated form of the recombinant hormone produced in bacterial cells is used in clinical trials. Similar to GM-CSF, G-CSF has also shown promising beneficial effects on chemotherapy. G-CSF reduces the myelotoxicity of chemotherapy, making the side-effects more tolerable. There is a reduction in the duration and the nadir of neutropenia after doxorubicin hydrochloride (Adriamycin), ifosfamide-mesna and etoposide treatment. G-CSF will increase the neutrophil count, reduce febrile episodes, and reduce the incidence of mucositis. However, only at very high doses will G-CSF increase monocyte or lymphocyte counts.

The significant consequence of G-CSF treatment in conjunction with chemotherapy in cancer patients was the partial mitigation of the myelosuppression. This permitted the completion of the cycle of chemotherapy without undue delay in patients being given chemotherapy for bladder cancer[1]. Myelosuppression was the cause for a lapse in chemotherapy in 70% of a control group who did not receive G-CSF[1]. However, this did not improve the survival rate or response rate of the patients who were administered G-CSF[1,7].

Treatment with G-CSF in bone-marrow transplant patients, as well as in patients with myelodysplasia, showed similar results as those treated with GM-CSF. The analysis of the advantage of using G-CSF or GM-CSF has not yet been fully explained, however. Further studies and clinical tests must be conducted before differences in the action of these 2 growth factors in such situations can be determined[1,7].

Idiopathic neutropenias are also being treated with G-CSF. For example, patients with Kostmann's Syndrome who have recurrent infections with considerable morbidity and mortality as a consequence, show improved neutrophil counts of 1,000 cells per mm^3 within 2 weeks after G-CSF administration. This level was sustained throughout continued therapy. The resolution of the preexisting chronic infection and a decrease in the number of subsequent re-infections also took place under treatment with G-CSF[1,7].

In addition, human cyclic neutropenia, a rare disorder in which periodic decreases in the number of neutrophils in the bone marrow and the blood occur, when treated with G-CSF showed a reduction in the length of neutropenia. G-CSF reduced both the severity of the disease and the accompanying symptoms. However, it did not rid the patient of the disorder. G-CSF as treatment has shown encouraging results in acquired chronic neutropenias. Obviously, further clinical trials and study are necessary to elucidate the potential benefits of administering G-CSF[1,7].

Conclusion

Research in the last 10 years has provided the information necessary to clone and to express the hematopoietic hormones. This has allowed for a wide range of clinical trials to determine the therapeutic benefits of these hormones in the treatment of various disorders. Erythropoietin increases the number of red cells in circulation. It has been utilized successfully in patients with renal disorders and anemias. In preoperative and postoperative patients, erythropoietin has resulted in more rapid red cell recovery. Erythropoietin is also employed to combat the side effects of various therapies, most notably in AIDS.

The colony-stimulating factors, IL-3, GM-CSF, and G-CSF, increase the number of leukocytes in vitro. The synergistic effects of these hormones requires extensive study in order to define the singular role each factor plays on the production of the desired cell types. Clinical trials are taking place to determine the effects of these factors when administered to patients with conditions involving decreased immune response as a result of low levels of circulating leukocytes. Of the 3 colony-stimulating factors discussed above, the least known for its therapeutic benefit is IL-3.

GM-CSF has also been beneficial when used in conjunction with various treatments, such as chemotherapy and AZT, by increasing the number of circulating neutrophils and monocytes. GM-CSF reduces the risk for both the donor and the recipient in bone marrow transplants. It resolves the anemias associated with a decrease in the marrow stem-cells, or it improves the marrow environment. It ameliorates the neutropenia resulting from therapy for cancer.

G-CSF has shown therapeutic benefits similar to GM-CSF. Bone-marrow transplants, myelodysplasia and neutropenias have been improved as indicated by increased numbers of neutrophils. In the treatment of the neutropenia caused by chemotherapy, G-CSF has shown advantages similar to those seen in the administration of GM-CSF. GM-CSF and G-CSF have shown similar results when utilized in clinical trials. The difference between the 2 growth factors has not yet been identified, further research and experimentation are needed to determine the advantage of one factor over the other.

The hematopoietic growth factors have the potential to enhance the quality of life in patients suffering from a broad range of illnesses and conditions. Defining the indications and the toxicities of these factors will be an area of enormous interest and importance in the years to come.

REFERENCES

1. Groopman JE, Molina JM, Scadden DT. Hematopoietic Growth Factors: Biology and Clinical Applications. *NEJM* 321:1449-1459.
2. Clark SC, Kamen R. The Human Hematopoietic Colony-Stimulating Factors. *Science* 236:1229-1236.
3. Sieff CA. Hematopoietic Growth Factors. *Journal Clinical Investigation* 79:1549-1557.
4. Golde DW, Gasson JC. Hormones That Stimulate the Growth of Blood Cells. *Scientific American* 259:62-70.
5. Dinarello CA, Mier JW. Lymphokines. *NEJM* 317:940-945.
6. Druker, B , Mamon HJ, Roberts TM. Oncogenes, Growth Factors, and Signal Transduction. Seminars in Medicine of the Beth Israel Hospital, Boston. 321:13831390.
7. Griffin JD. 1990. Colony-Stimulating Factors: A Review. New York, NY: Triclinica Communications, Inc.
8. Walker F, Nicola N, Metcalf D, Burgess A. Hierarchical Down-Modulation of Hematopoietic Growth Factor Receptors. *Cell* 43:269-276.
9. Waterfield MD. Growth Factor Receptors. *British Medical Bulletin* 45:541-553.
10. Bronchud MH, Dexter TM. Clinical Use of Growth Factors. *British Medical Bulletin* 45:590-599.
11. Johnson GR. Erythropoietin. *British Medical Bulletin* 45:506-514.
12. Dexter TM. Haemopoietic Growth Factors. *British Medical Bulletin* 45:337-340.
13. Sief CA, Emerson SG,Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC. Human Recombinant Granulocyte-Macrophage Colony-Stimulating Factor: A Multilineage Hematopoietic. *Science* 230:1171-1173.
14. Wong GG, Witek JS, Temple PA, Wilkens DM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC. Human GM-CSF: Molecular Cloning of the Complementary DNA and Purification of the Natural and Recombinant Proteins. *Science* 228:810-815.

■