

Human granulocyte-macrophage colony-stimulating factor: The protein and its current & emerging applications

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Blood cells under a variety of conditions produce cytokines which regulate their proliferation, communication and functioning. The granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor mainly responsible for the proliferation of granulocyte-s and macrophages. It is a molecule of immense therapeutic interest due to its wide-ranging actions on immune cells. Recombinant human GM-CSF has been expressed, purified and characterized. It has diverse biological manifestations, which has led to its being explored for use in cancer therapy, AIDS therapy, as a vaccine adjuvant, in certain types of wound healing, promotion of collateral growth of coronary artery, usefulness in treating Crohn's disease symptoms and in the management of a host of viral, bacterial and fungal infections. Newer therapeutic applications are also being unveiled. The physico-chemical as well as the biological features and the applications (current and emerging) of GM-CSF are discussed.

Keywords: GM-CSF, cytokines, T-cell induction, cytokines applications, GM-CSF applications, Crohn's disease

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Introduction

Most of the circulating cells of blood in humans are short lived and constantly need replacement throughout the lifespan. This process of blood formation is termed as hematopoiesis and is highly complex¹ owing to different types of cells that must be produced. Hematopoiesis² is capable of bringing about rapid adjustments in the number of cell sub-sets in the admixture to aid in a wide variety of conditions arising out of blood loss, infections, or as a result of the side effects of cytotoxic drugs/diseases. All different types of cells arise from a small pool of pluripotent stem cells in response to specific stimuli. Extremely precise and tight multi-point overlapping mechanisms are in place to ensure fidelity in normal conditions³. Derangement of this process results in various unhealthy conditions ranging from anemia to leukemia.

The pluripotent stem cells, in response to various chemical stimuli divide, differentiate and mature into specific cell sub-sets. These substances for stimulation are produced by cells under a variety of situations and stress conditions for the maintenance of homeostasis in the immune system⁴. These substances

secreted from cells are in general called 'cytokines' and they can have autocrine or paracrine modes of action. A wide spectrum of such substances are produced and are classified depending upon the type of cells they act on to produce the desired function, such as interleukins acting between leucocytes and lymphokines, which are secreted by lymphocytes or monokines that are associated with monocytes and macrophages⁵. Diverse range of cytokines regulates the intensity and the duration of the immune response by stimulating or inhibiting activation, proliferation and/or differentiation of the cells involved in the generation of humoral immune responses and also in the secretion of antibodies or other cytokines⁶. In case of cell mediated immunity, the hematopoietic progenitor cells differentiate into functional immune cells and their differentiation and proliferation also is regulated by different cytokines.

The pleiotropic nature of cytokines causes different cell types to secrete the same cytokine and a single cytokine can act on different cells, predominantly in a cascade fashion where one cytokine stimulates its target cells to make additional cytokines and more than one cytokine causes particular effector function⁷.

Cytokines that support growth and proliferation of hematopoietic colonies into blood cells are called colony-stimulating factors (CSFs)⁸. CSFs are acidic glycoproteins and are classified on the basis of the

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types of mature blood cells found in the resulting colonies^{9,12}. They include:

- Interleukin 3 (IL-3) — Stimulates stem cells to produce all forms of hemopoietic cells.
- Macrophage colony-stimulating factor (M-CSF) — Acts on cells of macrophage lineage.
- Granulocyte-colony-stimulating factor (G-CSF) — Acts on cells of granulocyte-lineage.
- Granulocyte-macrophage colony-stimulating factor (GM-CSF) — Affects the proliferation and differentiation of erythroid, megakaryocytic and myeloid lineage.

The life span of most blood cells is very short. If the body is not able to produce new healthy blood cells at the required rates, life-threatening infections or bleeding can occur. Such conditions can also be created due to cancer chemotherapy, in the case of bone marrow transplantation and other adverse clinical conditions. The growth promoting activities of these CSFs extend beyond their usual scope when they act in combination with other factors^{3,7}.

GM-CSF

Among the above mentioned major cytokines, GM-CSF has a special position for its therapeutic use in combating certain life threatening ailments. Several new uses of this cytokine are also expected to emerge in the near future. GM-CSF enhances the number of cells of monocyte and macrophage series in cancer patients¹³. It also enhances the ability of these cells to lyse tumour cells^{14,15}. In melanoma therapy, GM-CSF was found to act as an adjuvant¹⁶. It was also found to be the most potent immunomodulator for presenting tumour antigen to the immune system through the dendritic cells. Tumour cells engineered to secrete GM-CSF were used with success for vaccinating mice for suppressing tumour cells¹⁷ in animals through the activation of cytotoxic T cells and NK cells. GM-CSF is an efficient adjuvant and has a low toxicity profile. For the treatment of cancer, a large pool of data has been generated in animal models where the beneficiary effects of GM-CSF have been noticed¹⁸. It is one of the most important mediators of inflammation and has been used to improve the immunological functions of recipients, suffering from chronic viral hepatitis and certain hematological disorders¹⁹. GM-CSF contributes more towards accelerated production of populations of defense cells macrophages, granulocytes and eosinophils in states

of infection and inflammation than maintaining a steady state condition of population of defense cells. GM-CSF would fall in the category of "Black Cats" of our defense regimen of molecules to encounter the emergency situations. Because of these useful properties, it is anticipated that the interest of the biotech industry in this substance shall increase.

The GM-CSF protein was first purified in 1977 from mouse lung-conditioned medium using conventional chromatography and analyzed for structure and colony-stimulating properties²⁰. The protein of murine origin was cloned in 1984 for the first time²¹. Later, GM-CSF proteins of human and mouse origin were purified from mouse lung conditioned medium²². The gene for human GM-CSF was identified and cloned from the cDNA library of a T lymphoblast cell line (Mo). The recombinant human GM-CSF protein (rhGM-CSF) was transiently expressed in COS cells and was found to have all the biological functions when compared with purified natural human GM-CSF²³. Recombinant hGM-CSF has thereafter been cloned, expressed and purified in various other expression systems including Chinese hamster ovary (CHO) cells²⁴, *Pichia pastoris*²⁵, *Saccharomyces cerevisiae*²⁶, *Escherichia coli*^{27,28}, sugarcane²⁹, transgenic tomato suspension cultures³⁰; the resulting recombinant proteins have been characterized for their properties and biological activity.

The human *gm-csf* gene is approximately 2.5 kilo base pairs long. The gene is located on the long arm of chromosome 5 (gene locus 5q21-q32) as a single copy³¹. It has 4 exons that are separated by 3 introns. Besides the *gm-csf* gene, genes for IL-4, IL-5, IL-9, both subunits of IL-12, macrophage-CSF (M-CSF), c-fms (M-CSF receptor), EGR-1 (early growth response gene), IRF-1 (interferon regulatory factor-1) are located at the 5q31.1 locus of chromosome 5^{32,33}. The human *il-3* gene is located only 9 kb away from the *gm-csf* gene^{34,35}. The close arrangement of the genes encoding various cytokines and their receptor on chromosome 5 and the study of chromosomal aberrations resulting in interstitial deletions of parts of the long arm of human chromosome 5 (the 5q minus syndrome) has elucidated the role of these cytokines in the development of clonal hematopoietic stem cells³⁶.

The human GM-CSF (hGM-CSF) is synthesized as a 144 amino acid residue precursor protein with a 17 amino acid signal peptide. This precursor protein is

processed to yield a 127 amino acid mature protein with a predicted molecular mass of 14.4 kDa^{37,38}. The *gm-csf* gene and amino acid sequence of hGM-CSF protein are elucidated in Fig 1.

The apparent molecular mass of the natural GM-CSF has been found to be in the range from 14.5 to 32 kDa due to varying degree of glycosylation at six positions with both O-linked and N-linked carbohydrate structures^{24,39}. The isoelectric focusing studies and MALDI-TOF analysis of GM-CSF expressed in an engineered CHO cell line showed the existence of 16 different isoforms of the rhGM-CSF molecule²⁴. The different isoforms had differing numbers of carbohydrate moieties. The heterogeneity of glycosylation leads to wide variation in the apparent molecular weight of the native protein.

Natural GM-CSF has O-linked glycosylation sites at amino acid residues 22, 24, 26 and 27 and N-linked glycosylation sites at amino acid residues 44 and 54³⁷. Interestingly, it was observed that glycosylation is not required for biological activity of the protein and the removal of carbohydrates actually increased the specific activity of GM-CSF³⁹. Receptor binding studies demonstrated lower receptor affinity for the heavily glycosylated GM-CSF molecules as compared to the less glycosylated forms or non-glycosylated GM-CSF molecules produced in *E. coli* due to differences in kinetic association rates⁴⁰. It has also been demonstrated that the presence of N-linked carbohydrate significantly inhibits *in vitro* biological activity of GM-CSF. Studies with hGM-CSF mutants having reduced glycosylation also gave similar conclusions⁴¹. It is further speculated that

glycosylation of GM-CSF may be involved in increasing the half-life of the molecule in serum by increasing its stability. The hGM-CSF has four cysteine residues that form two disulfide bonds between cys54-cys96 and cys88-cys121⁴². The disulfide bridge between cys55 and cys96 is essential for the biological activity; the one between cys88 and cys121 could be removed without significant loss of activity^{43,44}. Physical studies carried out on recombinant GM-CSF show that it is a compact, monomeric, globular protein, which is hydrophobic in nature^{44,45}. Crystal structure of rhGM-CSF as determined by X-ray crystallography⁴⁶, shows that the protein is made up of four α -helices (42%)⁴⁴ that pack together and two antiparallel β -sheets (57%)⁴⁰. The schematic structure of hGM-CSF protein is presented in Fig. 2. The four α helices labeled as A, B, C and D are each about 11-15 amino acid residues long. Helix A spans amino acid residues 13-27, helix B spans residues 55-65, helix C has residues 74-86 and helix D has residues 103-116. The helices pair as A with D and B with C, which run antiparallel to each other. The connections between A and B helices span amino acid residues from number 28 to 54, those between B and C helices are amino acids 66 to 73, and those between C and D helices are 87 to 102 (Fig. 2). Two long over hand connections link helix A to B and helix C to D. Within these connections, residues 42-44 (S1) and 99-101 (S2) form a twostranded antiparallel β -sheet^{44,48}.

Experiments with hybrid GM-CSF molecules containing cross species regions and competition assays showed that the residues 95-111 and 38-48 are

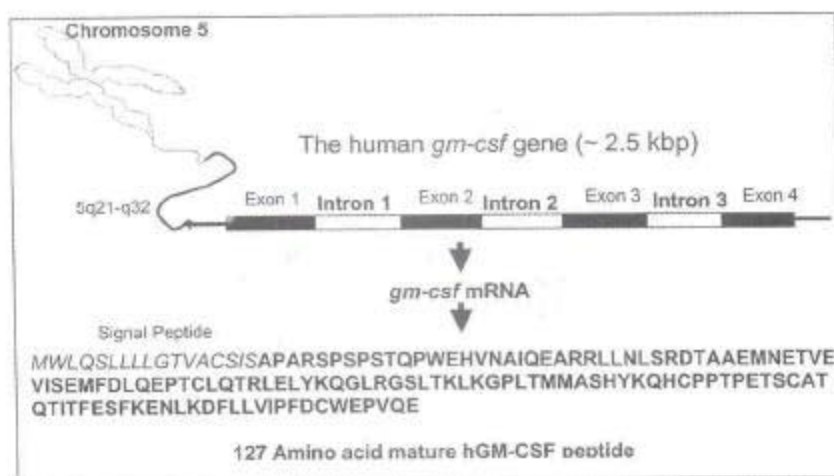


Fig. 1 — Schematic representation of the *gm-csf* gene location, structure and sequence of the 17 amino acid long signal peptide followed by the 127 amino acid mature hGM-CSF peptide.

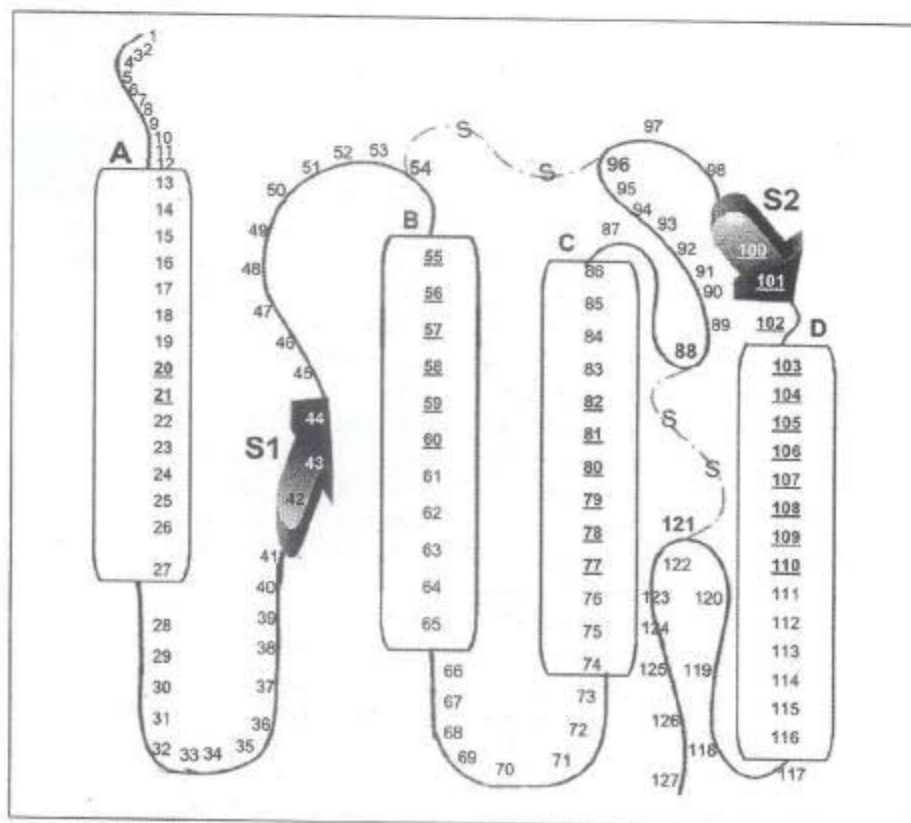


Fig. 2 — Schematic representation of the hGM-CSF molecule showing the four α -chains (A, B, C and D), the two β -sheets (S1 and S2) as well as the two disulphide bonds. The amino acid numbers of the protein shown as bold and underline were identified to be critical for biological activity 46,47. The protein has alanine as the starting amino acid (marked 1) and glutamic acid (127) as the last one.

critical for hematopoietic functions⁴⁹. Studies using synthetic fragments of GM-CSF have shown that amino acids 1-13 at N-terminus are not important for rhGM-CSF activity while the integrity of residues 16, 17 and 18 is essential for biological activity⁵⁰. Experiments using chemically synthesized GM-CSF peptides show that amino acids 14-25 are required for activity and amino acids 97-121 are important for full activity⁵¹. Epitope mapping studies using neutralizing monoclonal antibodies have suggested that amino acids 77-94, 40-94 and 110-127 are necessary for receptor binding and/or full activity. Amino acids 21-31 and 78-94 that are parts of the α -helices A and D respectively, are found to be critical for hematopoietic function because the α -helices A and D form the major contact region with the GM-CSF receptor⁵². Studies based on scanning deletion analysis show that amino acids 20-21, 55-60, 77-82 and 89-120 are critical for activity/structural integrity of GM-CSF⁵³. Studies using synthetic peptides and neutralizing monoclonal antibodies showed that the

integrity of residues 14-42, especially 16-18 was critical for biological activity and the synthetic peptide of GM-CSF having residues 31-113 is active biologically⁵³. This information indicates that most of the structure of the GM-CSF molecule is required for receptor binding for complete biological activity. Homology studies and analysis of biological activities have shown that GM-CSF proteins of different animal origins share a high degree of sequence homology but are absolutely species specific for activity^{53,54}. Amino acids 77-82 contribute to the species-specific character of the GM-CSF molecule. This was determined by the substitution of human critical region with mouse residues⁵³.

Expression of GM-CSF and its Regulation

GM-CSF is synthesized by a variety of cell types in response to specific activating signals such as inflammatory agents (lipopolysaccharides, FCS, low density lipoproteins), infection causing agents (antigens), monokines such as IL-1 and tumour

necrosis factor (TNF) and in general, in response to any kind of immune challenge⁵⁵. Tumour cells and lymphomas are also known to secrete the cytokine. GM-CSF is further reported to be secreted in synovium arthritis and by acute myeloid lymphoma cells (Table 1).

T cells, macrophages, mast cells, endothelial cells and fibroblasts on induction accumulate GM-CSF mRNA and secrete GM-CSF protein. Expression is regulated by both transcriptional and post-transcriptional control mechanisms. The *gm-csf* gene is constitutively transcribed in monocytes, endothelial cells and fibroblasts without accumulation of mRNA. When these cells are activated by a variety of stimuli, transcription of GM-CSF mRNA increases and the mRNA gets stabilized⁶². This leads to transient mRNA accumulation in the cells. GM-CSF mRNA has an untranslated 62 base pair AT rich sequence found to be responsible for the instability of the mRNA. The *gm-csf* gene has a AU-rich element (ARE) that controls translation and stability of the mRNA by affecting the poly A-binding protein mediated mRNA circularization. The GM-CSF is known to be responsible for mRNA instability and poor mRNA translation⁶⁷. The *gm-csf* gene expression is also found to be regulated by a 'TATA' like sequence found to be present at 20-22 bp upstream of the transcription initiation site and a homologous region extending 330 bp upstream the TATA box^{38,68}. Site directed mutagenesis studies show that hGM-CSF gene is activated by the transcription factors NFAT and AP-1 dependent enhancer. This enhancer has four NFAT binding sites with a AP-1 binding site⁶⁹. Therefore, the GM-CSF enhancer activity is induced in cell types that express NFAT eg; T-cells, myeloid cells and endothelial cells⁷⁰. External stimuli such as antigens and endotoxins influence increased GM-CSF production.

GM-CSF Receptor (GMR)

The hGM-CSF is found to have a broad range of effects on several different types of cells, which include monocytes, megakaryocytes, eosinophils, blast cells, uterine cells as well as macrophage progenitor cells^{32,71,72}. The effect of cytokines on various cell types is mediated through the interaction of the cytokines with specific high affinity receptors that are expressed on the cells^{73,74}. Since receptor for each of the colony-stimulating factors are unique, cells that respond to more than one cytokine express on their surface more than one type of CSF receptor.

Table 1 — Cells Expressing GM-CSF

Cell Type	Stimuli	Reference
T-Lymphocytes	Antigen, lectin, IL-1, HTLV	23, 56
B-Lymphocytes	Lipopolysaccharides(LPS), TPA	57
Macrophages	LPS, phagocytosis, adherence	55, 58
Mast cells	IgE	59
Fibroblasts	Tumour Necrosis Factor (TNF), IL-1, TPA	60-62
Endothelial cells	TNF, IL-1, TPA	60, 63, 64
Mesothelial cells	Epidermal Growth Factor + TNF	65
Osteoblasts	Para Thyroid Hormone, LPS	66

The hGM-CSF receptor (GMR or CD 116) is species specific⁷⁵. In humans, GM-CSF, IL-3 and IL-5 mediate several common biological functions and exhibit cross competition for receptor binding which indicates that they may share same receptor or receptor subunits^{71,76,77}. The GM-CSF receptor consists of two different subunits, the α -chain (GMR α) and the β -chain (β c)⁷⁸. The GMR α , which is required for cytokine specific signaling, is the major binding contact and the β c, which is common with IL-3 and IL-5, is the major signal transducer^{78,79}. Both α and β subunits of GMR are required for complete signaling. Both the cytoplasmic and trans membrane components of the receptor are required for signal transduction by GM-CSF. Exposure of one CSF could cause the down regulation of other receptors on the cell surface. Also the mean number of receptors on a normal responsive cell varies i.e. receptors for G-CSF, GM-CSF and multi-CSF are 100-500/cell while receptors for M-CSF are 3000-16000/cell. This difference in distribution could be due to the better proliferative effects of G-CSF and GM-CSF as well as the short half-life of the M-CSF receptor complex. The expression of common subunits of the receptors for IL-3, IL-5 and GM-CSF are regulated by alternative mRNA splicing⁷⁹. The specific regions of the GM-CSF α -helix are defined for the formation of GM-CSF receptor complex. Binding of GM-CSF to GMR α is due to specific interaction between Asp112 in helix D of GM-CSF and Arg280 in GMR α loop⁸⁰. Amino acid Glu21 in helix A is required specifically for high affinity binding. This has been established by substitution of the amino acid to generate GM-CSF analogs which are found to bind with low affinity and lack complete biological activity^{81,82}. Studies employing site directed mutagenesis of key residues on the GMR α , indicated that His-367 is critical for high affinity binding of the receptor to Glu-21 of hGM-CSF proteins⁸³.

Experimental studies using soluble receptor components of GM-CSF that were cloned in a suitable vector from cDNA and a GM-CSF antagonist showed that the assembled ternary complex of GM-CSF and its receptor (GMR) comprises GM-CSF molecule associated with one molecule of GMR α which interacts with a non-covalently linked dimer of the β c⁸⁴. This ternary complex is known to signal to the cell resulting in a biological response through the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway, Phosphatidylinositol 3-kinase (PI-3 kinase) pathway and the ras/mitogen activated protein (MAP) kinase pathway⁸⁵. The GM-CSF-GMR complex has a proline rich region in the β c, which is the binding site for JAK2⁸⁶. The cytoplasmic domain of the β c has multiple tyrosine residues that are targets for phosphorylation that recruit the proteins STAT1, STAT3 and STAT5 to form a DNA binding complex. This in turn causes the induction of *c-myc* and activation of DNA replication. GM-CSF binding to GMR also causes the activation of *ras* and mitogen activated protein kinases, which leads to induction of the genes (*c-fos*, *c-jun*) involved in regulation of hematopoietic differentiation. Similarly upon activation, the *ras*/MAP kinase and PI-3 kinase act in a cascade to affect the cytokine function on the cell⁸⁵. Figure 3 gives a schematic representation of these interactions.

Biological Functions/Effects of GM-CSF

Initial *in vitro* studies using purified GM-CSF indicated that this cytokine is involved in clonal proliferation, differentiation and survival of myeloid precursors into neutrophilic and eosinophilic granulocytes and monocytes and acts as a potent stimulator². Molecular cloning and production of large quantities of the recombinant protein made it possible to study its uses in clinical settings^{87,88}. Early experiments suggested that under certain culture conditions, GM-CSF could also cause proliferation of erythroid burst-forming units (BFU-E)^{89,90}. In addition to cell proliferation, purified protein was also demonstrated to enhance the effector functions of mature cells of the immune system^{91,92}.

Studies using transgenic *gm-csf* knock-out mice showed the capacity of GM-CSF to act *in vivo* as a regulator of hematopoiesis^{93,94}. These mice, although had normal survival time, fertility and leukocyte counts, were more prone to bacterial and fungal infections compared to the normal animals. These findings are in contrast to the effect of G-CSF gene

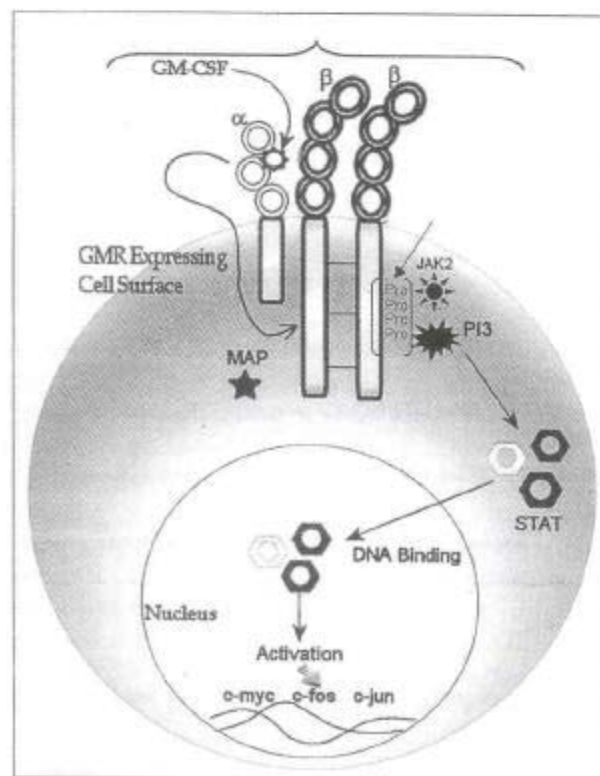


Fig. 3 — The GM-CSF and its receptor (GMR) forms a ternary complex with binding of GM-CSF to the α -subunit which interacts to activate a non-covalently linked dimer of the β c84. This ternary complex signals to the cell resulting in a biological response through the JAK/STAT pathway. The proline rich region of the β c of GMR is the binding site for JAK2 which recruits STAT proteins. They form a DNA binding complex that in turn activates the *c-myc* gene and DNA replication. On activation, PI-3 kinase and MAP kinase also act in a similar cascade to affect the cytokine function of the cell.

knock-out which results in chronic neutropenia and impaired hematopoiesis in stress conditions such as infections^{95,96}. Interestingly, however, GM-CSF deficient animals developed chronic pulmonary disorders and were susceptible to pulmonary infections^{97,98}. In humans, GM-CSF plays a role in regulating pulmonary surfactant homeostasis and in the pathogenesis of inflammatory lung disease, acute respiratory distress syndrome (ARDS) and lung fibrosis⁹⁹.

The cytokine exerts both direct and indirect effects on human neutrophil functions, such as inhibition of neutrophil migration, degranulation, changes, in receptor expression profile, and 'priming effects' to enhance the ability of neutrophils to respond to secondary triggering stimuli for example, increase in

superoxide and leukotriene synthesis¹⁰⁰⁻¹⁰². GM-CSF is also reported to enhance chemotactic, antifungal and antiparasitic activities of the mature granulocytes and monocytes^{91,103}, as well as the cytotoxicity of monocytes against neoplastic cell lines and activation of polymorphonuclear neutrophils to inhibit the growth of tumour cells *in vitro*^{13,104}. It is also involved in the proliferation of several other cells of the immune system in combination with other CSFs such as with erythropoietin to promote proliferation of erythroid progenitors and with IL-3 to promote proliferation and differentiation of myeloid progenitors^{2,7,82,105}. It acts on monocytes/macrophages to enhance phagocytosis, antibody dependent cytotoxic activity and antifungal activity. Besides these, GM-CSF has several other biological functions/effects which include interaction with receptors on mature neutrophils, monocytes and eosinophils, to inhibit apoptosis and prolong their survival, increase adhesion, affect their motility to attract and accumulate them at the site of inflammation, enhance phagocytosis, cause degranulation and antibody dependent cell cytotoxicity^{88,106,107}. GM-CSF also induces macrophages to produce other cytokines such as G-CSF and monocytes to produce M-CSF, promotes differentiation of Langerhan's cells into dendritic cells (DCs) and helps to recruit DCs into tumour cells^{32,108}. It enhances the migration and proliferation of endothelial cells and promotes keratinocyte growth¹⁰⁹. The physiological functions and biological effects on the cells of immune system have suggested that GM-CSF could be an interesting drug for the correction of neutropenic conditions arising due to infections and other clinical conditions.

Present and Promising Future of rh-GM-CSF in Therapy

Recombinant human GM-CSF (rhGM-CSF) is one of the most clinically successful products for application among the biological therapeutic agents. Use of Sargramostim (yeast derived rhGM-CSF) and Molgramostim (bacteria derived rhGM-CSF) has been approved for management of neutropenic conditions in the induction chemotherapy in acute myelogenous leukemia (AML) to shorten the time to neutrophil recovery and reduce the incidence of severe and life-threatening conditions. The recommended dose of rhGM-CSF for use in AML patients is 250 µg/m²/d over a 4 h i.v. transfusion¹⁰⁹. In a clinical trial involving 99 patients (52 treated with GM-CSF and 47 in the placebo group), administration of rhGM-CSF significantly enhanced the hematologic recovery and reduced the incidence of severe infection, death from pneumonia and fungal infections^{109,110}. Use of rhGM-CSF has been found to be beneficial for patients undergoing autologous and allogeneic bone marrow transplantation and, for mobilization and engraftment of peripheral blood progenitor cells in a variety of cancer patients^{109,111}.

After the elucidation of the molecular structure of rhGM-CSF, recombinant DNA technology has been employed to synthesize biologically active rhGM-CSF molecules in different expression systems. Several rhGM-CSF formulations are commercially available (Table 2)¹¹²⁻¹¹⁵.

With the generation of data from the ongoing trials, the clinical indications for the use of rhGM-CSF are increasing considerably giving the drug a promising future owing to its diverse biological effects. Proliferative role of rhGM-CSF on progenitor cells of

Table 2 — rhGM-CSF formulations

Name	Expression host	Glycosylation	Recommended dose	Presentation
Sargramostim (Leukine®) (Amino acid sequence identical to hGM-CSF except for Leu ²³ in place of Pro ²³)	<i>Saccharomyces cerevisiae</i> (yeast)	O-glycosylated (glycosylation may differ from hGM-CSF)	250 mcg/m ² /d	Leukine liquid vial (500 mcg, 2.8×10 ⁶ IU): sterile injectable solution. Leukine lyophilized vial (250 mcg, 1.4×10 ⁶ IU)
Molgramostim (Leucomax®) (Amino acid sequence identical to hGM-CSF)	<i>Escherichia coli</i> (bacterial)	Non-glycosylated	5-10 mcg/kg body weight/d	Leucomax lyophilized vial (300 mcg)
Regramostim (none)	<i>Chinese hamster ovary</i> (mammalian)	Fully glycosylated	-	-

neutrophils, eosinophils and monocytes has been established since its administration causes increase in peripheral blood counts of these immune cells. It is, therefore, being used extensively in the treatment of neutropenia. It also is found to modulate the functions of the immune cells that includes enhancement in oxidative metabolism, cytotoxicity and phagocytosis in monocytes and macrophages, enhanced cell maturation and migration in dendritic cells, increase in surface adhesion molecules (β integrins, Fc receptors and complement receptors) on neutrophils to enhance defense against fungal and bacterial infections. The rhGM-CSF effects increase in number of class II Major histocompatibility complex (MHC) on the surfaces of macrophages and dendritic cells thereby enhancing their antigen presentation capacities^{116,117}.

The rhGM-CSF has been shown to activate and enhance the phagocytic ability of neutrophils and their ability to destroy infection causing bacteria and fungi such as *Staphylococcus aureus*¹¹⁸, *Candida albicans*¹¹⁹, *Aspergillus fumigatus* and *Trypanosoma cruzi*¹²⁰. Significant growth inhibition of *Mycobacterium* complex has been reported in GM-CSF treated human macrophages as well as in a mouse model of disseminated *Mycobacterium avium* complex^{121,122}.

GM-CSF is reported to have been used in clinical trials in HIV patients undergoing antiretroviral therapy for amelioration of drug-induced myelosuppression. Potential uses of the cytokine have been evaluated in the light of the accumulating evidence that it has microbicidal and antiparasitic activities. Initial *in vitro* studies had raised doubts that the GM-CSF might be activating the HIV replication, however, three clinical trials reported suppression of HIV expression by rhGM-CSF as long as the patients received concurrent antiretroviral therapy¹²³⁻¹²⁵. The HIV-positive patients receiving rhGM-CSF adjunctive therapy along with HIV protease inhibitor drugs showed significant increase in the CD4⁺ cell counts and concomitant decrease in the viral load. A possible explanation for reduction in expression of HIV upon treatment with rhGM-CSF was provided in studies where it was elucidated that the expression of the β -chemokine receptor CCR5 (co-receptor for virus attachment and entry) was downregulated by GM-CSF¹²⁶. Another possible use for rhGM-CSF in HIV patients is in the management of opportunistic infections due to its microbicidal activity. These *in vitro* observations are highly encouraging for the

possible use of this protein in HIV therapy but the results may need further clinical evaluation.

Interesting applications of GM-CSF are emerging in the area of vaccine development. GM-CSF is beyond doubt an important cytokine for the generation and propagation of DCs, the antigen presenting cells that play an important role in the induction and sustenance of primary and secondary immune responses. DCs display antigens on their surface in conjunction with class II MHC. The rhGM-CSF also augments expression of MHC class II molecules on the DC surface¹²⁷. By enhancing antigen presentation to the lymphocytes and inducing expression of other cytokines such as IL-1, IL-6 and TNF, rhGM-CSF causes augmentation of cellular immune response against vaccine antigens. Experiments in animals using protein and peptide-based vaccines with GM-CSF as an adjuvant indeed showed that the immunogenicity of the vaccines was increased many-fold compared with other traditional adjuvants approved for human use¹⁸. Trials are underway, where rhGM-CSF is being used along with recombinant canary-pox virus HIV vaccine to confirm the adjuvant like effect of rhGM-CSF in humans¹²⁸. These studies will monitor augmentation of humoral as well as cellular immune responses against the vaccine antigen and the role of the cytokine. Several studies have found that the use of GM-CSF as an adjuvant enhanced the immunogenicity of DNA vaccines in terms of humoral as well as cellular immune responses. Several other preliminary studies using rhGM-CSF in conjunction with Hepatitis B vaccine and tetravalent Influenza vaccines have shown good results and suggest that rhGM-CSF may be a good adjuvant for antiviral vaccines. More trials, however, are needed to establish the efficacy and safety of rhGM-CSF for use as an adjuvant in humans^{17,129}.

Since rhGM-CSF enhances antibody dependent cellular toxicity of immune cells it has potential application for antitumour therapy along with monoclonal antibodies. The rhGM-CSF is being increasingly used for treatment of various diseases including cancer and complications of chemoradiotherapy such as mucositis (mouth sores), stomatitis and diarrhoea¹¹⁶. Topical application of rhGM-CSF has found to accelerate wound healing with increased formation of granulation tissue¹³⁰. Intradermal injection of rhGM-CSF causes enlargement of keratinocytes, thickening of epidermis and

enhancement in healing¹³¹. A short-term use of GM-CSF only in the subcutaneous mode was effective in promoting the growth of collateral coronary artery in patients suffering from coronary artery diseases¹³². The protein has also been found useful in improving Crohn's disease symptoms¹³³.

Commercial Production of rhGM-CSF — A Strategy

Considering the huge therapeutic potential of GM-CSF, development of strategies for production of this protein at low costs is desirable so that the drug is made available to a vast population. Following the cloning of the human GM-CSF gene, the biosynthetic hGM-CSF has been produced and purified from yeast, mammalian cells and bacteria (*E. coli*)¹³⁴. Several strategies for the preparation of pure rhGM-CSF have been described in literature and patents that use reverse phase high pressure liquid chromatography (RPHPLC) and column chromatography combinations to achieve pure GM-CSF.

We propose the use of a simple method that we have standardized for the preparation of the active rhGM-CSF protein using a two-step column chromatography. We have expressed the protein as inclusion body in *E. coli* host. The culture conditions have been optimized for a high glucose medium that takes care of basal GM-CSF expression which is known to be toxic to the host cells and also gives a high biomass OD up to 7.0 in a shake flask. A batch of 6L medium in 12 specifically designed baffled shake flasks gives about 12g/L cell pellet. The cell biomass is processed for extraction and partial purification of inclusion body protein using a simple process that involves sonication followed by washing by detergents at low concentration.

Cell pellet from a 6L batch yields about 60 mg of IB protein, which has about 50 mg of GM-CSF protein. The GM-CSF protein is solubilized out from this preparation using low concentration of denaturants. The solubilized protein is allowed to refold in oxidizing conditions using standard renaturants like L-arginine. The renatured protein is desalted by dialysis and the protein processed to adjust the pH of the solution. This processing eliminates a majority of the contaminating host cell proteins from the preparation. The protein is then purified to >99% using two ion exchange chromatography columns. The protein is characterized and formulated. The typical yield from this process is about 10% of the starting inclusion body protein preparation. The general strategy of the

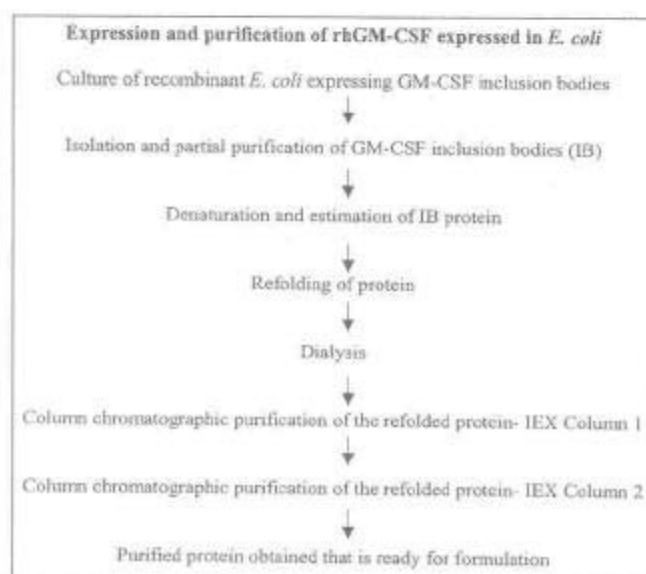


Fig. 4—Schematic flow chart depicting the essential steps followed in our process of expression and purification of rhGM-CSF.

process is depicted in Fig 4. The purified protein matches the specifications laid down for purity and physico-chemical characterization of the standardized protein preparations. We shall publish these results separately.

Concluding Remarks

The cytokine GM-CSF is found to mediate biological actions at a molecular level through receptor signaling by activation of kinases by cellular phosphorylation and the resulting expression of several mitotic genes including the nuclear oncogenes such as *c-myc*, *c-jun* and *c-fos*¹³⁵ in a regulated manner. These proteins further affect hematopoiesis by controlling cell growth and vitality as well as apoptotic events through complex mechanisms that are of extreme interest for study in the scientific community¹³⁶. One of the mechanisms to affect apoptosis is found to work through the activation of PI-3 kinase which acts through a cascade like mechanism to activate apoptosis suppressor protein *Bad*. Several such mechanisms and cascades might exist, which would be better understood with more research on those lines.

GM-CSF and a group of other cytokines such as G-CSF, M-CSF and IL-3 are involved in regulation of cellular hematopoiesis and the control of growth and activities of blood cells with each cytokine having more than one biological effect. Also, these cytokines have diverse and overlapping functions; therefore it

can be understood that they must act in a complex network¹³⁷. Considering that GM-CSF is found to mediate such a wide range of actions, more is the understanding of cellular biochemistry better will be our ability to exploit this wonder molecule of the body in therapeutic applications.

The human GM-CSF protein with apparent molecular mass varying between 14.5 and 32 kDa due to its different degrees of glycosylation at the six probable positions (four O-linked and two N-linked) makes the product complex in its natural form. Interestingly, the non-glycosylated protein expressed in *E. coli* is also biologically active. This is also the case with the product expressed in yeast with different kinds of glycosylation. The biological activities of the natural product and the non-glycosylated recombinant product expressed in *E. coli* show certain differences; indeed, the naturally occurring product is less active than the non-glycosylated or less glycosylated product. It is not yet clear if these differently glycosylated genuine, human derived products have finer individual differential roles to play in modulating the growth and proliferation of hematopoietic colonies. The future years may only unveil such aspects. In the present scenario, the interest in the recombinant molecule is increasing because of its demonstrated therapeutic applications in a variety of life threatening situations including neutropenia in patients receiving cancer chemotherapy and its strong adjuvant actions in enhancing susceptibility of cancer cells to the cytotoxic T-cells and NK cells. GM-CSF also mediates inflammation caused under different stress situations including impaired respiratory conditions. Patients suffering from chronic viral hepatitis shall be benefited from the use of GM-CSF.

The natural product is difficult to isolate and has limitations in availability. The recombinant DNA technology is reasonably well established and holds promise towards large availability at affordable prices. It is anticipated that with increased interest in the molecule by different research groups the biologically active, stringently purified, stable formulations of rhGM-CSF shall be available in large quantities during the future years all over the world for the benefit of mankind.

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