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Review Article

HEMATOPOIETIC GROWTH FACTORS AS BIOPHARMACEUTICALS: AN OVERVIEW

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ABSTRACT

For the past four decades, the application of recombinant DNA technology and other novel allied technologies has opened the door for production of many proteins in large quantities that have become important new biopharmaceuticals for the treatment of many diseases. Hematopoietic growth factors (HGFs) are among the newer biopharmaceuticals that have approved and commercialized for the treatment of different types of diseases. The HGFs including erythropoietin (EPO), granulocyte macrophage colony-stimulating factor (GMCSF), granulocyte colony-stimulating factor (GCSF), macrophage colony-stimulating factor (MCSF), thrombopoietin (TPO) and interleukin-3 (IL-3) have been known for over 25 years, named for their role in the proliferation, differentiation and survival of hematopoietic progenitor cells. Especially, the recombinant forms of EPO, GMCSF and GCSF have been used for many years in clinical practice in oncological and hematological pathology. Recent studies suggest that HGFs have also important non-hematopoietic functions in the brain, heart, kidney and other organs. This review briefly summarizes the different physiological functions of the major HGFs. It also provides a critical and comprehensive overview of the different therapeutic applications of the recombinant forms of some of the HGFs.

Keywords: Biopharmaceuticals, Hematopoietic growth factors, Erythropoietin, Granulocyte macrophage colony-stimulating factor, Granulocyte colony-stimulating factor, Macrophage colony-stimulating factor, Thrombopoietin, Interleukin-3.

INTRODUCTION

The discovery of novel technologies such as genetic engineering and hybridoma technology in 1970s[1] and recent advances in our understanding of disease biology, biomarkers, new therapeutic targets, and innovative modalities have each fueled a dramatic expansion in the development of novel biopharmaceuticals.[2] Such novel therapeutic agents that are also referred to as biotechnology-derived pharmaceuticals, biotherapeutics, biologics or biotech drugs can now be developed with high degree of selectivity and affinity for their intended targets.[2, 3] It is now 30 years since the approval of the first biopharmaceutical for general medical use ('humulin', recombinant human insulin produced in *Escherichia coli*, initially approved in 1982).[1, 4, 5] The

pharmaceutical biotechnology industry has matured rapidly in the intervening years. Today, there are more than 220 such products in general medical use and several hundred are in the pipeline.[5] The global market value estimates vary depending upon source and exactly how you define a biopharmaceutical, but generally the global biopharmaceutical market is expected to increase to 167 billion US Dollars by 2015.[6]

Currently, the biopharmaceuticals encompass peptides, proteins, glycoproteins and nucleic acid-based biologicals that are used for therapeutic, prophylactic or *in vivo* diagnostic purposes and are produced by means other than direct extraction from a native (non-engineered) biological source.[7, 8] They are generally high molecular weight products of biological origin that are complex, with a molecular composition that is difficult to define since they are derived from heterogeneous mixtures made from the products of living organisms, cells, animals or plants. Most are produced by genetic engineering and few by hybridoma technology rather than a series of known, controlled chemical reactions. The successful development and production of biopharmaceuticals requires scientific innovation, manufacturing skills, broad interdisciplinary knowledge and a large investment. Well understood purification techniques are also essential. Major pharmaceutical companies are rapidly developing and/or acquiring expertise in the development and production of biopharmaceuticals.[6] For example, the development of novel techniques such as site-directed mutagenesis and other protein engineering approaches, along with an increased understanding of protein structural–functional relationships, facilitates the development of proteins of altered amino acid sequence tailored to better fulfill pre-specified therapeutic goals. Different therapeutic proteins that have been engineered in this way achieved several potential therapeutic outcomes.[9] Now a days a substantial part of the FDA approved drugs [8] and those that are being developed to fight cancer, infectious diseases, genetic disorders and different chronic diseases include enzymes,[10, 11] vaccines and monoclonal antibodies,[10,12,13] hormones, clotting factors, thrombolytic agents,[7, 14] engineered cell or tissue-based products,[5] nucleic acid based drugs such as antisense drugs [9,10] and cytokines such as interleukins (ILs), interferons (INFs) and HGFs [10, 11, 15] belong to the biopharmaceuticals. Among the numerous biopharmaceuticals that had been already approved for medical use and that are under development, this review will focus specifically on those that play a significant role in hematopoiesis. Hematopoiesis is the development of progenitor and mature blood cells from immature, pluripotent, long term reconstituting hematopoietic stem cells (HSC) located in the bone marrow. [16] The production of blood cells is a tightly regulated process involving an interacting network between various inhibitory and stimulatory cytokines presented by the immediate microenvironment or HSC niche and their corresponding receptors expressed on HSC. These pleiotropic molecules have specific effects on the pathways that regulate cellular behavior, interactions, communication, and death, either directly and/or by regulating expression of other cytokines.[16, 17] A number of pleiotropic glycoproteins that act, either as soluble molecules released from cells, or as cellular membrane-bound ligands to regulate aspects of hematopoiesis have been identified. Some of the most influential cytokines includes colony-stimulating factors (CSFs), which are known to be the most important HGFs,[16, 18] such as GMCSF, [19] GCSF, [20, 21, 22] MCSF,[23, 24] and multi-CSF (or IL-3).[18] These factors show both functional pleiotropy, exhibiting a wide variety of biological functions on various tissues and cells, as well as significant redundancy, being able to exert similar and overlapping functions on specific cells. Overall, the CSFs mediate the survival, proliferation, differentiation and functional modulation (chemotaxis, degranulation, activation, adhesion, cytotoxicity, mRNA phenotype changes) of

various populations of mature blood cells and their precursors.[17, 25] In addition to the CSFs, hormones such as TPO and EPO that play potent regulatory roles in the development of some hematopoietic cells from pluripotent stem cells are important HGFs.[16, 26] TPO, the ligand for the cytokine receptor c-Mpl, is a naturally occurring glycosylated peptide growth factor and the primary regulator of megakaryocytopoiesis. Activation of c-Mpl stimulates the differentiation of bone marrow stem cells into megakaryocyte progenitor cells, promoting megakaryocyte proliferation and maturation and increasing the number of platelets in the peripheral blood.[27, 28] EPO is also a glycoprotein hormone, synthesized by the mammalian kidney in response to hypoxia and released into the circulation to stimulate the maturation and differentiation of erythroid red blood cell precursors. The action of EPO is mediated via a specific surface receptor, located in small numbers on the erythroid progenitor cells.[29, 30] This review will provide a critical and comprehensive overview of the different HGFs that are used as biopharmaceuticals for the treatment of different diseases.

HEMATOPOIETIC GROWTH FACTORS AS BIOPHARMACEUTICALS

Blood is one of the most highly regenerative tissues, with approximately one trillion (10^{12}) cells arising daily in adult human bone marrow.[31] The mature hematopoietic or blood cells, which are vital to human life, consists of a variety of cells responsible for oxygen transport (red blood cells), hemostasis (platelets derived from megakaryocytes), innate immunity against infections(granulocytes,monocytes/macrophages and mast cells), and acquired immunity (T and B lymphocytes).[32] These cells are derived from small numbers of self- renewing pluripotent HSCs cells that reside in the bone marrow and generate progenitor cells committed to proceed along one of the maturation pathways. Because the life span of blood cells is limited, the production rate of blood cells in the marrow is high, even during steady state conditions. The marrow system has the ability to adapt to sudden changes in the needs of different cell compartments by elevating the production rate of blood cells of specific cell lineages. To satisfy these variable needs, a tight control of the processes of cell renewal, commitment, maturation, and survival for each of the differentiation stages within each blood cell lineage is required. The HGFs play a critical role in regulating these processes. [33]

HGFs represent a family of hormone like, pleiotropic glycoproteins that can regulate both hematopoiesis and the functional activity of mature blood cells. The former includes the differentiation, proliferation and survival of precursor cells and maturation of the hematopoietic cells. In addition, HGFs mobilize progenitor cells to move from the bone marrow to the peripheral blood.[34] Most of the HGFs are glycoproteins which can be distinguished by their amino acid sequence and glycosylation (or carbohydrate linkages). They have cysteine-cysteine disulfide bridges that dictate their three-dimensional configuration that is necessary for biologic activity.[35] The HGFs that are required for the survival and proliferation of hematopoietic cells at all stages of development are produced by different cells in humans. T lymphocytes, monocytes or macrophages, fibroblasts and endothelial cells are the major cellular sources of most HGFs except for EPO and TPO that are mainly produced by liver and kidney cells.[36, 37] Many inflammatory stimuli are capable of promoting the cellular release of HGFs. Antigens, lectins and IL-1 can signal T lymphocytes to produce GMCSF and IL-3. Lipopolysaccharides such as endotoxins can induce monocytes and macrophages to release GCSF and GMCSF. Monocytes can produce MCSF after stimulation by the products of activated T lymphocytes such as INF- γ, IL-3 and GMCSF or after exposure to tumor necrosis factor-alpha (TNF- α). IL-1 and TNF- α that are produced by activated monocytes can trigger the release of GCSF and GMCSF by fibroblasts and

endothelial cells.[38] Proliferation and differentiation of progenitor cells to become mature blood cells requires intimate contact between stem cells, stromal cells and the extracellular matrix, and is mediated by the HGFs. [39] HGFs act by binding to specific cell surface receptors. The resultant complex sends a signal to the cell to express gene, which in turn induce cellular proliferation, differentiation or activation.[34, 40] The name of most of each HGF is derived from its predominant target cell. Moreover, the HGFs, on the basis of their action, are characterized either as multi-lineage hematopoietins, e.g., stem cell factor or as lineage restricted hematopoietins, e.g., GCSF, MCSF, EPO and TPO. [39] Each of these HGFs support the survival and proliferation of a number of distinct target cells, and the elimination of any one of them does little harm because of the redundancy in the functions of most of these glycoproteins.[36] Generally, HGFs support a wide array of physiologic functions. For example, GMCSF, in conjunction with GCSF, IL-5 and MCSF, respectively, supports the proliferation and differentiation of neutrophil, eosinophil and monocyte precursors, and directly stimulates their mature progeny to become functionally activated. Furthermore, GMCSF acts to promote differentiation and survival of peripheral blood dendritic cells, augments the primary antibody response by enhancing function of antigen presenting cells, activates endothelial cells to proliferate and migrate, and together with EPO directly stimulates the proliferation and differentiation of intermediate and late erythroid progenitor cells.[40] Different studies have also shown the non-hematopoietic functions of different HGFs. For example, the use of GMCSF as adjuvants in HIV DNA vaccine development is one of the functions of this growth factor beyond hematopoiesis.[41 - 43] Some HGFs and their receptors are expressed by neurons in many brain regions and are up-regulated after focal ischemia, indicating an autocrine protective response of the injured brain. The neuroprotective function of HGFs has been suggested by the effect of decreasing infarct volumes in different experimental models in rodents and has been attributed to their anti-apoptotic activity. Moreover, HGF induces neurogenesis and angiogenesis, possible the substrate of improving recovery post-stroke. There is emerging data from different studies suggesting that EPO, GCSF and GMCSF are potential new agents, a novel type of multi-factorial drugs and candidates for neuroprotection in ischemic stroke.[44] Especially, EPO has functions beyond erythropoiesis to ameliorate different diseases of the brain, heart and other organs.[41 - 43]

For the past four decades, the application of recombinant DNA technology using microbes and other cell lines has opened the door for the availability of multitude of new biopharmaceuticals for the treatment of many diseases. Novel biotherapeutics are increasingly making their way into clinical applications. Today more than 220 approved peptide, protein and glycoprotein based recombinant pharmaceuticals are on the FDA list for general medical use and several hundred are in the pipeline.[5, 45] Among the biopharmaceuticals, the recombinant versions of three HGFs (as shown in table 1) are commercially available for clinical use as therapeutic agents for the treatment of many clinical disorders involving different types of blood cells.[46] The recombinant versions of other HGFs are also under development. The advents of recombinant DNA technology and allied technologies have introduced a variety of new strategies for modulating the properties of proteins such as efficacy, stability, specificity, immunogenicity and pharmacokinetics. The strategies for altering these properties include manipulation of primary structure, conjugation or incorporation of fusion partners and post translational modifications. The controlled manipulation of the physical, chemical and biological properties of proteins enabled by structure-based simulation is now being used to

refine established rational engineering approaches and to advance new strategies.[47] For example; glycosylated forms of most HGFs might have greater clinical efficiency *in vivo*. Studies had shown that glycosylated GMCSF to have a longer serum half life, greater neutrophil stimulating activity, less leukotriene production and fewer side effects than the non-glycosylated GMCSF preparation.[48] Although there are numerous HGFs that play a significant role in hematopoiesis and in other physiological functions beyond hematopoiesis, this review focuses on those growth factors that are produced by recombinant DNA technology and marketed in Europe and USA such as EPO GCSF and GMCSF. Other HGFs such as MCSF, TPO and IL-3 will be also briefly discussed in this review article.

Table 1: Clinical uses of some recombinant HGFs[46]

HGFs	Commercial name	Approved applications
EPO	Eprex® (Epoetin- α) Epogen® (Epoetin-ß)	Anemia, Reduction of allogenic blood transfusion in surgery patients
GCSF	Neupogen®(Filgrastim) Neulasta® (Pegfilgrastim)	Acute myeloid leukemia, Severe chronic neutropenia
GMCSF	Leukine® (Sargramostim)	Acute myelogenous leukemia; Myeloid recovery after autologous & allogenic bone marrow transplantation
	Macrogen® (Molgramostim)	Severe neutropenia

Erythropoietin

EPO is a glycoprotein with 166 amino acids with carbohydrate linkages, i.e. three –N-linked glycosylations and one-O-linked glycosylation.[49] This glycoprotein with a molecular weight of 30.4 KDa has a predominant role in red blood cell production.[50] The EPO gene is located on chromosome 7, encoding for a polypeptide chain containing sequences of amino acids. EPO is synthesized by renal peritubular cells in adults and by hepatic cells in the fetus; a small amount is also synthesized in the adult liver.[49, 50] In addition to kidney and liver, the brain tissue and other cells in our body synthesize and release EPO.[51] Tissue oxygen demand and oxygen transport capacity regulate EPO production and secretion.[50] For example, the expression of EPO in fetal liver and adult kidney is generally hypoxia inducible and is regulated via the hypoxic response element in the 3' region of the EPO gene, and the reporter genes exhibit trans-activation by the dimeric hypoxia inducible factor (HIF) -1.[52] The EPO cDNA can be associated with a "strong" promoter, physiologically regulated by oxygen tension and containing one or several copies of hypoxia responsive element (HRE) consensus sequence.[53] The modulation of EPO gene expression in many organs by hypoxia led to the hypothesis that oxygen sensing is a general phenomenon and there fore wide spread and found in other organ as well.[54] Different studies had shown the regulatory role of the hypoxia inducible transcription factors (HIFs) on EPO gene expression. Both HIF – Iα and its isoform, HIF-2α are considered to be the master regulators because of their involvement in regulating several important pathways such as angiogenic, glycolytic and survival pathways.[55] Comparisons of HIF- Iα with its isoform, HIF-2α using small interfering RNA technology suggested that HIF-2α rather than HIF-1α is the main regulatory of EPO gene expression during hypoxia.[52] In addition, different endogenous chemicals play significant roles in regulating EPO production and secretion. For instance, both nor-epinephrine and epinephrine and many of the prostaglandins also stimulate EPO production.[56] Many clinical and experimental studies have demonstrated that EPO, as a multifunctional tropic factor, has

different sites of expression, a tissues specific regulation and several mechanisms of action.[57]

The diverse physiological actions of EPO is associated with a functional EPO receptor that is present in hematopoietic progenitor cells and is also expressed in non-hematopoietic systems, such as endothelial cells, myocardial cells, smooth muscle cells, prostatic cells and peripheral and central nerve cells.[50] The EPO receptor is one of class 1 cytokine receptors that signals through member of the Janus family of cytoplasmic tyrosine kinase 2 and signal transducers and activators of transcription 5 (STAT5).[58] After EPO binds to its receptor, many of the cell types exhibit a specific biological reaction via the activation of intracellular biological pathways.[50] For example, EPO mediates erythropoiesis by binding to its specific receptor expressed on the surface of immature erythroblasts.[59] The expression of EPO receptor on a broad array of non-hematopoietic systems has opened the door to conduct extensive research to the non-hematopoietic effects of EPO. EPO generally modulates a broad array of cellular processes that include progenitor stem cell development, cellular integrity, causing vasoconstriction-dependent hypertension, increasing serum rennin, stimulating angiogenesis, and stimulating the proliferation of smooth muscle fibers and vessel endothelium. It is emerging as a cell death blocker and a vascular growth factor with promising protective potential in the setting of acute and chronic myocardial ischemia and may potentially represent a powerful pharmacological addendum in the fight against cardiovascular, brain and other diseases.[50, 60]

Physiological roles of erythropoietin

Erythropoietic effect

The term erythropoiesis has been used to describe collectively the erythropoietic cellular pathway, composed of all cells involved in erythropoiesis, starting with the earliest committed erythroid progenitor and ending with mature circulating red blood cells.[61] It has been more than a century, in 1906, since *Carnot and DeFlandre* first announced the existence of a circulating erythropoietic factor. Fifty years later EPO was discovered and the kidneys, more specifically the interstitial peritubular cells, were established as the predominant site of production.[62] EPO is the chief regulator of erythropoiesis, and is required for survival, proliferation and differentiation of committed erythroid progenitor cells in the bone marrow.[63] EPO provides essential survival signals to allow the proper terminal differentiation of red cell precursors. The erythropoietic action of EPO is mediated by activation of the EPO receptor that is expressed on erythroid progenitor cells via homodimerization.[58, 64] The EPO receptor does not contain a kinase domain and signaling is mediated by the interaction of the intracellular domain with effector molecules such as Janus kinase 2 (JAK2) and STAT5.[64] The binding of EPO to its receptor induces a conformational change of the homodimeric- EPO receptor and triggers JAK2 phosphorylation and activation. Phosphorylation of JAK2, in turn, phosphorylates several tyrosine residues such as STAT5 on EPO receptor providing docking sites for binding of several intracellular proteins and activation of multiple intracellular signal transduction pathways [64, 65, 66] that ultimately inhibiting apoptosis of erythroid precursor cells and supporting their proliferation and differentiation into normoblasts.[67]

Neuroprotective effect

For several years EPO has been believed to act exclusively on erythroid precursor cells; however several lines of evidences suggest a potential role other than erythropoiesis.[68, 69] First of such evidences is that both EPO and its specific receptor are expressed in different tissues, including the nervous system.[69] In the central nervous system, the EPO gene is expressed in the temporal cortex, amygdala, and hippocampus. It has been hypothesized that EPO and its receptor are prominent in the brain during

fetal development, leading to speculation that they play an important role in neurodevelopment and in brain homeostasis.[70, 71] More over, over the last 10 years, a wide variety of experimental studies have shown that EPO exerts a remarkable neuroprotection in both cell cultures and in animal models of nervous system disorders.[65, 69] The neuroprotective actions of EPO and its underlying mechanisms in terms of signal transduction pathways have been defined and there is a growing interest in the potential therapeutic use of EPO for neuroprotection.[68, 72]

EPO has been reported to induce a broad range of cellular responses in the brain directed to protect and repair tissue damage during ischemic and traumatic brain conditions. A fundamental mechanism of EPO-induced neuroprotection is its ability to inhibit apoptosis through promotion of cell survival signaling cascades and up regulation of the expression of anti-apoptotic proteins. [72,73] The other mechanisms of EPO-induced neuroprotection include anti-inflammatory, modulation of intracellular calcium metabolism, attenuation of nitric oxide production, inhibition of glutamate release, anti-oxidant, angiogenic, anti-epileptic and neurotrophic effects. [71-73] The protective function of EPO in neurons is generally mediated by activation of JAK2.[51, 72] It is not easy to differentiate each mechanism distinctly. To better understand these mechanisms a summary of EPO signaling pathways in neuronal protection has been demonstrated in Figure 1.[72]

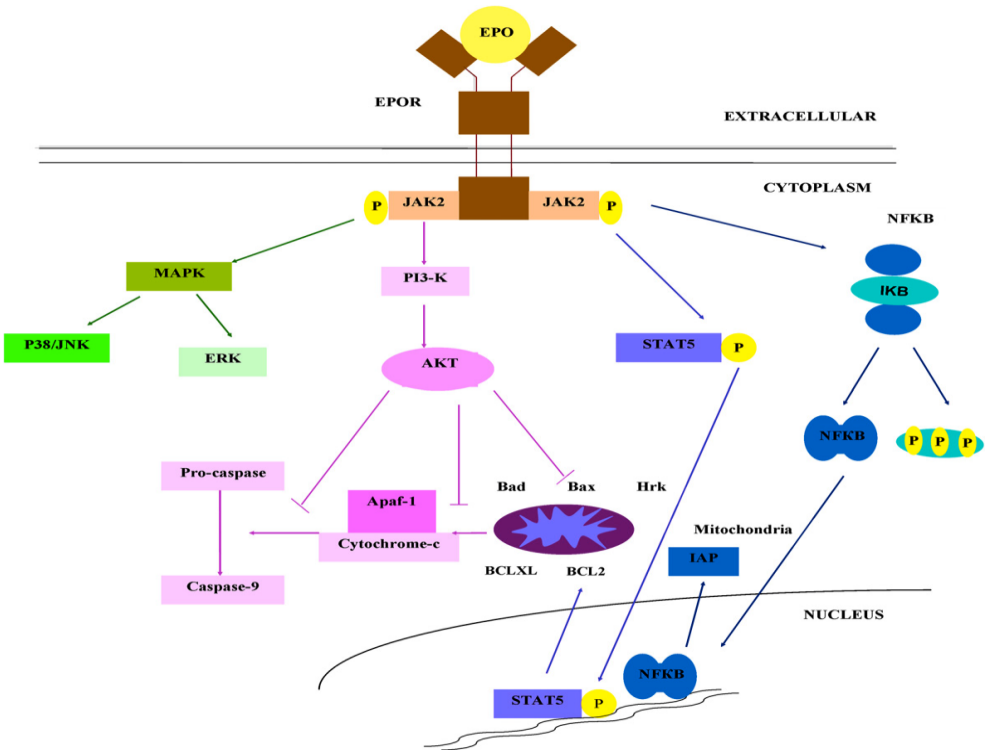

Figure 1: Summary of EPO signaling in neuronal cell. EPO binds to EPO receptor dimer and stimulates JAK2 kinase activity which results in phosphorylation of JAK2 and EPO receptor. Activated JAK2 initiates signal transduction through several downstream molecules such as STAT-5, mitogen-activated protein kinase (MAPK), ERK, phosphatidylinositol-3-kinase (PI3- K)/AKT and IKB. Nuclear factor-kB (NFkB) dissociates from IKB. NFkB and STAT5 enter into the nucleus, bind to DNA, and transcribe neuroprotective genes such as Bcl-xL and bcl2.[72]

Angiogenic effect

Angiogenesis is the process of the formation of new blood vessels from pre-existing ones.[74] It is a complex process that normally occurs under tight regulation in adults only under specific conditions such as wound healing, inflammation and development of the corpus luteum in the

menstrual cycle. Under normal conditions such as wound healing, the angiogenic process switches on and then off at the appropriate times indicating tight regulation of stimulatory and inhibitory factors.[75, 76] The potential roles of EPO and EPO receptor in vascular function have been indicated in both *in vitro* and *in vivo* studies. EPO has been shown to increase microvascular branch formation from rat aortic rings in a standard angiogenic assay. In addition, EPO has been shown to up-regulate expression of several genes involved in vascular function, signal transduction and energy transfer, in cultured endothelial cells.[72] Numerous studies have established a crucial pro-angiogenic role of EPO, as indicated by enhanced mobilization of progenitor cells, to elicit vascular repair.[66] In angiogenesis, EPO stimulates proliferation of endothelial progenitor cells (EPCs), production of matrix metalloproteinase-2, migration of endothelial cells into vascular sites, and formation of capillary tubes.[72] Stimulation of EPCs by EPO is an importance component of EPO activity in vascular injury.[52] It has been shown that stimulation of cultured endothelial cells with EPO resulted in cell proliferation, chemotaxis, and differentiation into vascular structures. Furthermore, it had been found that EPO and vascular endothelial growth factor were equally effective in stimulating angiogenesis in endothelial cells derived from the myocardium.[77]

Cardioprotective effect

EPO and its receptor have been shown to be present in the heart. Several reports have revealed cardioprotective effects by EPO against myocardial ischemia or reperfusion injury and heart failure.[78] Recently, numerous *ex vivo* and *in vivo* studies have shown a protective role of EPO during cardiac ischemia.[79] Administration of EPO at the time of cardiac ischemia will have positive effect that it is cardioprotective by preventing apoptosis and stimulating angiogenesis. [80]

Effect on nitric oxide production

Beyond its role in vascular regulation, a substantial body of evidences suggested that vascular nitric oxide has additional function in the maintenance of vascular homeostasis based on its anti-inflammatory, antiatherogenic, and anti-apoptotic effects as well as anti-platelet properties.[81] EPO inhibits inducible nitric oxide synthase expression and prevents nitric oxide production in excess amount and protecting the neuron from death. Nitric oxide acts as a neuromodulator and a neurotransmitter in the central nervous system, participating in synaptogenesis, memory formation and endocrine secretion. However, excess nitric oxide seems to be important mediator for neuro-destructive effects. When nitric oxide reacts with superoxide, a more deadly nitrite, peroxynitrite, is formed, which is a potent oxidant and nitrating agent capable of attacking and modifying proteins, lipids and nucleic acids, depleting antioxidant defenses, and finally results in neuron death.[82]

Anti-oxidant effect

Oxygen free radicals are produced at low levels during normal physiological conditions and are scavenged by endogenous anti-oxidant systems that include superoxide dismutase, glutathione peroxidase, catalase and small molecule substances such as vitamins C and vitamin E. EPO controls a variety of signal transduction pathways during oxidative stress that can involve JAK2, protein kinase B, signal transducer and activator of transcription pathways, mammalian fork head transcription factors, caspases, and NFkB.[72, 83] *E. Ozturk et al.* have demonstrated that prophylactic single dose administration of EPO protect oxidative stress – induced brain traumatic injury.[84] EPO reduced lipid peroxidation by both decreasing nitric oxide synthesis and xanthine oxidase activity, and by increasing the activities of cytosolic anti-oxidant enzymes such as superoxide dismutase and glutathione peroxidase.[85]

Anti-inflammatory effect

Several studies have investigated the ability of EPO to affect inflammatory responses. It is thought to have anti-inflammatory activity.[86] In the hearts of mice with myocardial infraction, the levels of inflammatory mediator cytokines such as IL-1β (IL - 1β), IL-6, TNF- α and transforming growth factor β1 (TGF - β1) were returned to nearly control level by EPO treatment.[81] It has also anti-inflammatory role during ischemia/ or reperfusion damages and trauma in different tissues as well as in central nervous system.[87] The exact mechanisms of the anti-inflammatory effects are unknown. But EPO might reduce leukocyte transmigration through endothelial cells, since EPO enhances the resistance of endothelial cells towards ischemia.[72]

Recombinant Erythropoietin as therapeutic biopharmaceuticals

The major breakthrough that transformed the therapeutic field of anemia management came in 1977 with successful purification of small amounts of human EPO from the urine of patients with aplastic anemia.[88] Based on limited sequence information of this purified material, the gene for human EPO was then isolated and cloned in 1983 and the use of genetic engineering techniques allowed the large scale production of recombinant human EPO in suitable mammalian cell lines called Chinese Hamster Ovary cells (CHO cells).[89] Now a days, it is possible to produce large quantities of highly purified EPO for pharmaceutical application by using recombinant DNA technology.[90] For large scale production of EPO, a gene coding for human EPO is cloned and the corresponding protein can be expressed in CHO cell lines in which recombinant CHO cells are frequently cultivated in roller bottle or in suspension.[91] In recent years, different expression host cells have been studied for production of recombinant human EPO. For example, *D. Kodama et al.* have successfully used chimeric chickens to produce recombinant human EPO in milligram quantities in egg white. Several other groups have also explored the possibility of producing recombinant human EPO in the milk of transgenic mammals, but the production level has been low.[92]

Neorecormon (trade name, also known as epoietin-ß) is one of the recombinant human EPO produced by recombinant DNA technology using a CHO cell line. It was first approved for medical use in Europe in 1997.[14] More recently, an engineered form of EPO has gained marketing approval. Such biosimilar EPOs includes Epoietin- α (Eprex®), Epoietin delta (Dynepro®) and Epoietin zeta (Ritacrit®) are erythropoiesis stimulating agents that have important role in treating anemia. Like other most glycoproteins, biosimilar EPOs are heterogeneous with respect to glycosylation, resulting in different isoforms. Although it is extremely difficult to establish the precise contribution of individual glycoforms to the overall activity, toxicity and immunogenecity of these biopharmaceuticals, analysis of their glycosylation pattern is of utmost importance in attempting such understanding to guarantee drug quality and efficacy.[93, 94] Moreover, the non-erythropoietic derivatives of EPO such asialo EPO [95] and carbamylated EPO [96] have been developed. They exhibit broad spectrum of non-erythropoietic property with reduced hematopoietic responses.[97]

The synthesis of various forms of recombinant human EPO represented a breakthrough in the treatment of anemia due to end stage renal disease, cancer, chemotherapy, arthritis, acquired immunodeficiency syndrome (AIDS), chronic heart failure-related anemia and anemia due to post partum hemorrhage.[67, 98] Experimental evidence from animal models of acute organ injury affecting the brain, heart, kidney and other organs have shown similar beneficial effects following administration of recombinant human EPO, through the activation of intracellular pathway, that determine cell fate in response to various conditions.[99] Recombinant human EPO and its analogues have promising therapeutic

potentials for treatment of brain diseases,[100,101] cardiovascular diseases such as acute myocardial ischemia [99, 102] and chronic heart failure, [103, 104] atrophic age-related macular degeneration[105] and acute renal failure.[106] However, the use fullness of these biopharmaceuticals is limited by some adverse effects such as hypertension,[107] cancer, seizures, arteriovenous fistula or shunt thrombosis, hyperkalemia[108] and immunogenicity. [109, 110] Many studies revealed the EPO limitation owing to its serious adverse effects, especially those due to immunogenic reactions of EPO.[110] Therefore, controlled and long-term studies of efficacy, safety and quality with novel formulations and delivery systems are required to establish sustainable clinical benefits from EPO and its analogues.

Granulocyte Macrophage Colony-Stimulating Factor

Neutrophilic granulocytes and monocytes /macrophages are phagocytic leukocytes derived from common myeloid progenitor cells.[111] They play an important role in host defense, both through their intrinsic action against invasive organisms and as part of the mechanisms that regulate the behavior of other immunocompetent cells. These dual characteristics of being not only the source but, simultaneously, the target of cytokines have opened the discussion concerning the real role of tissue structural cells within the immune response.[112] The different glycoproteins leading to the differentiation, production and activation of these phagocytic cells are referred to CSFs. GMCSF is one of the well characterized CSFs which affect the development of bone marrow precursor cells.[111]

GMCSF influences myelopoiesis by stimulating the differentiation of stem cells to produce granulocytes, monocytes and macrophages.[113, 114] In addition; it can stimulate accessory cell functions of granulocytes, monocytes, macrophages, eosinophils and neutrophils and contributes to the differentiation of monocytes toward dendritic cells. [114, 115] The maturation and functional activity of antigen presenting cells such as macrophages and dendritic cells is improved by GMCSF. This pleomorphic glycoprotein induces the migration of immature dendritic cells to the T-cell area of lymphoid organs while up-regulating the expression of MHC class II and co-stimulatory molecules, thereby improving the capacity of antigen presenting cells to prime naive T-cells.[116] It induces peripheral monocytosis and prolongs the life-span of monocytes via a reduction of apoptosis.[117] GMCSF stimulates monocytes to produce TNF- α, IL-1α and IL-1ß and primes neutrophils for enhanced chemotaxis, leukotriene B4 synthesis, arachidonic acid release, production of free radicals and cytotoxic activity.[112] In addition to hematopoietic cells, GMCSF stimulates migration and proliferation of human endothelial cells. Moreover, GMCSF is capable of inducing the development of osteoclasts and stimulating and regulating proliferation of normal human epidermal keratinocytes and human melanocytes.[118]

GMCSF is a 127 amino acid monomeric protein with 2 glycosylation sites formed from a 144 amino acid precursor. Structurally, GMCSF is a 4-helix-bundle glycosylated cytokine broadly similar in structure to growth factors such as IL-2, IL-3, IL-5 and GCSF.[119] GMCSF is generally an acidic glycoprotein (human = 18-22 kDa; mouse = 23 kDa) [118] that is secreted by activated T cells, endothelial cells, fibroblasts, mast cells, B cells, macrophages, monocytes, [111, 120] astrocytes,[121] and airway smooth muscle cells.[119] The receptor for GMCSF has been characterized and found to measure approximately 84 KDa. GMCSF receptor presence on both myeloid and non-myeloid cell membranes, including those of tumor cells, has been demonstrated.[122] GMCSF signals via a heterodimeric receptor, which composed of a specific α- chain and common ß-chain shared with IL-3 and IL-5 to exert its pleiotropic effects on cell differentiation, activation, survival and on inflammatory.[119]

GMCSF and other HGFs have been and continue to be evaluated in many clinical disorders involving different types of blood cells. Neutrophil disorders are a logical therapeutic target for the myeloid HGFs including GMCSF. Abnormal neutrophil function may occur because of defective adhesion, movement or phagocytosis and killing. Insufficient numbers of neutrophils or neutropenia may occur because of accelerated destruction, maldistribution or decreased production. In either case, patients generally have impaired host immunity and an increased risk of infection. Thus, the ability of GMCSF to stimulate proliferation of bone marrow progenitors in cells committed to myeloid differentiation has prompted considerable investigation and enthusiasm in the real and potential clinical applications of this HGF.[122]

The list of real and potential clinical applications of GMCSF is still expanding and may include: (1) correction of cytopenias after cancer chemotherapy and/or radiotherapy, (2) acceleration of hematopoietic recovery after bone marrow transplantation, (3) reduction of toxicity and acceleration of myelopoiesis which may conceivably allow increase in the dose of antineoplastic drugs, (4) increased mobilization, collection and transplantation of peripheral blood progenitor cells as an alternative to bone marrow transplantation, (5) *ex vivo* expansion of hematopoietic cells of bone marrow or blood origin, (6) direct stimulation of antitumor activity of granulocytes and monocytes, (7) application in the treatment of infectious diseases, (8) cancer gene therapy involving vaccination with turnor cells genetically altered to secrete GMCSF.[120, 123]

The recombinant human GMCSF (rhGMCSF) that is produced by recombinant DNA technology using *Escherichia coli* as expression host cells, molgramostim, is already marketed world-wide for clinical purposes. Molgramostim is a non-glycosylated polypeptide chain consisting of 127 amino acids, with a molecular mass approximately 14.5 kDa and with four cysteine residues which form two disulphide bonds, between Cys 54 and Cys 96 and Cys 88 and Cys 121.[114] The other rhGMCSF is sargramostim, which is a yeast-derived glycosylated protein with a molecular mass approximately 23 kDa.[124] One of the clinical applications of these biopharmaceuticals is to enhance reconstitution of hematopoietic functions and to reduce treatment-induced neutropenia associated with myelosuppressive cancer chemotherapy, bone marrow transplantation and antiviral therapy for AIDS related cytomegalovirus infection. The anticipated therapeutic benefits of rhGMCSF in this role are to reduce myelosuppression and clinically decrease the incidence of neutropenic sepsis, febrile morbidity and mortality, which are the principal side effects of a large number of chemotherapeutic agents.[122] rhGMCSF is also indicated for failed bone marrow transplantation or delayed engraftment, and for use in mobilization and following transplantation of autologous peripheral blood progenitor cells.[120, 125, 126] In addition to the existing therapeutic applications, the results of animal experiments and early clinical studies provided further support for the use of GMCSF as a vaccine adjuvant and demonstrated that GMCSF is a promising option for the immunotherapy of different infectious diseases and cancer because of its potential to eradicate disseminated disease without systemic toxicity.[127, 128] GMCSF protein has an adjuvant-like effect when co-administered with protein and peptide vaccines. Similarly, plasmids encoding GMCSF can act as `genetic adjuvants, boosting the immune response elicited by DNA vaccines.[116] Generally, GMCSF seems potentially very useful as molecular adjuvant for a variety of vaccines, including cell based vaccines, peptide and protein based vaccine and DNA vaccines.[129]

Granulocyte Colony-Stimulating Factor

Neutrophils are essential as a local barrier of host defense and are one of the first cells at the

site of injury and infection that possess an arsenal of potent antimicrobial responses for the elimination of infectious agents through granulocytic phagocytosis, chemotaxis and microbicidal activities.[130, 131] The potential toxic threat that neutrophils pose to host tissue should they undergo a spontaneous response is limited due to the short lifespan of neutrophils by pre-programmed apoptosis. Thus, the continuous production of neutrophils and the necessity of the immune system to respond to pathogens by increasing neutrophil numbers, must be tightly regulated.[131] It is primarily through the action of GCSF, which is a pleiotropic cytokine that promotes the growth, proliferation, differentiation and maturation of neutrophil precursors.[132, 133] It induces their terminal differentiation and enhances the function of mature neutrophils by increasing phagocytic activity and antibody-dependent cell-mediated cytotoxicity. [134, 135] Although GCSF was originally identified as a growth factor which specifically regulates proliferation and differentiation of neutrophilic granulocytes, it exhibited diverse biological activity beyond regulation of granulopoiesis. For example, it was shown to stimulate the growth of non-hematopoietic cells such as colon cancer cells, vascular endothelial cells, small cell lung cancer cells [136] and some myeloid leukemic cells.[137] GCSF also augments the release transforming growth factor -ß and platelet-derived growth factor, which are the endogenous mediators that in turn act on the fibroblasts to improve connective tissue regeneration through the acceleration of the formation of the mature collagen fibers. Moreover, GCSF induces the production of nitric oxide, another endogenous mediator, to further regulate connective tissue regeneration.[138] In addition, GCSF exerts a powerful neuroprotective effect in various types of neurological disorders such as stroke, neurotrauma and neurodegenerative diseases.[139]

GCSF is produced primarily by hematopoietic cells such as monocytes and macrophages.[111, 120] Several non-hematopoietic cell types, such as osteoblasts, smooth muscle cells, endothelial cells, epithelial cells, reproductive tissue cells, fibroblasts [120, 138, 140] and several malignant tumors [141] have also been shown to produce GCSF. GCSF exerts its biological effects through binding to specific, high-affinity GCSF receptors. GCSF receptors are members of the class I cytokine receptor superfamily that have been reported on hematopoietic cells of the granulocytic lineage, platelets, monocytes and lymphocytes.[131, 140] In addition, receptors for GCSF have been detected on non-hematopoietic cell types, including vascular endothelial cells, human placenta and trophoblastic cells, human myeloid leukemic cells and leukemic cell lines, oral, mesopharyngeal and bladder carcinoma cells, cell lines derived from human small cell carcinoma of the lung and cell lines derived from skin carcinoma.[140, 141] Like other members of class I cytokine receptor superfamily, the GCSF receptor lacks intrinsic tyrosine kinase activity, but its ligation results in the activation of cytoplasmic tyrosine kinases.[142] The active tyrosine kinases such as JAKs, JAK1 and Jak2, and tyrosine kinase-2 phosphorylate substrates, including the receptor, to provide docking sites for other proteins, which in turn, are phosphorylated as well. Proteins docking to the GCSF receptor complex include members of the STAT family. Upon tyrosine phosphorylation, STATs form dimeric complexes and translocate to the nucleus, where they influence gene transcription. Of the six members of the STAT family identified in mammalian cells, STAT1, STAT3 and STAT5 have been implicated in GCSF signaling. [139, 142] These signaling pathways ultimately lead to the migration, survival, proliferation, and differentiation of neutrophils and the action of GCSF on some non-hematopoietic target cells.[131] GCSF is a glycoprotein consisting of 174 amino acids and a single O-linked glycosylation site and has a molecular weight of 19.6 KDa. The gene encoding GCSF is located on human chromosome 17.[14, 136] In an attempt to obtain

human GCSF in large quantity, the gene encoding it has been cloned, and the recombinant human GCSF had been successfully expressed in and purified to homogeneity from CHO cells and *Escherichia coli*, and was approved for clinical use in 1991.[132, 143] Two forms of recombinant human GCSF are currently available for clinical use: a glycosylated form obtained by expression in CHO cells (such as lenograstim) and a non-glycosylated form synthesized in an *Escherichia coli* expression system (such as filgrastim).[144] Neupogen (trade name also known as filgrastim) is a recombinant human GCSF protein expressed in inclusion bodies in *Escherichia coli* by Amgen. It has 175 amino acids, with a molecular weight of 18.8 KDa. The protein has an amino acid sequence that is identical to the natural human sequence, except for the addition of an N-terminal methionine due to the cytoplasmic expression strategy used in *Escherichia coli*. Because Neupogen is produced in *Escherichia coli*, the product is non-glycosylated and thus differs from GCSF isolated from a human cell.[14, 134, 144] Although there is a huge market demand for Neupogen, economical large scale production of this biopharmaceutical is still a challenge with respect to biosynthesis and downstream processing, especially in the *Escherichia coli* system, due to partitioning of the expressed protein as insoluble material into inclusion bodies that requires solubilization and renaturation steps during purification. More recently, a high throughput, parallel processing approach to expression strain engineering was used to evaluate soluble expression of human GCSF in *Pseudomonas fluorescens*. The production of soluble GCSF in the periplasm of *Pseudomonas fluorescens* would be advantageous for downstream processing because the disulfide bond containing protein should be properly folded, thereby requiring no renaturation steps during purification.[144] The biological activities of filgrastim, lenograstim and pegfilgrastim were similar to those of the endogenous human GCSF. Therefore, recombinant forms of human GCSF have been commercialized for their clinical uses.[143, 145] They are indicated for neutropenia associated with myelosuppressive cancer chemotherapy, bone marrow transplantation and severe chronic neutropenia.[146, 147] They are also indicated to mobilize peripheral blood progenitor cells for autologous stem cell transplantation[148] after high-dose chemotherapy and for reversal of clinically significant neutropenia and subsequent maintenance of adequate neutrophil counts in patients during infections.[133, 143,149, 150] It is also suggested that GCSF might be chosen as a first line therapeutic strategy in the treatment of accidental acute radiation exposed victims.[144] Several studies have highlighted the promise of recombinant human GCSF as a possible therapy for cerebrovascular disease, [151] amyotrophic lateral sclerosis, [132] and for brain, heart, liver and kidney injuries induced by a variety of pathological conditions. [152] Recombinant GCSF is one of the neuroprotectants showing promise for the treatment of different neurological disorders. Its pharmacological and side effect profile is well known since it is already licensed for use in other indications in humans.[151]

Generally GCSF has few side effects; some studies have indicated that GCSF has the potential to enhance the lung toxicity of pneumotoxic agents such as the bleomycin and to cause harmful effects on the lung even in the absence of known pneumotoxic drugs. In some instances, activated neutrophils have been implicated in the pathogenesis of microvascular injury in the lung, resulting in adult respiratory distress syndrome. [135] GCSF can also cause medullary bone pain as the major side effect in approximately 10 to 20 % of patients.[143] Further more; GCSF is not suitable for outpatient use due to its intrinsic instability [132] and its short circulation half life. [143] Thus, it should be excessively and/or frequently administered to patients in order to maintain a plasma concentration which is high enough to achieve therapeutic effects. This administration regimen

causes inconvenience and pain in patients.[132, 153] Different strategies had been used to enhance the biological activity and stability of recombinant human GCSFs. For example, the mutant and PEGylated derivatives of recombinant human GCSF had been developed. These derivatives had shown longer circulation half life and better stability when they compared with unmodified recombinant GCSF. [154] Thus, the development of GCSF derivatives through site directed mutagenesis and site specific PEGylation is one of the potential approaches that have to be further explored in order to increase the biological half life and stability and to reduce the immunogenicity GCSF. It is hoped that the next generation of GCSF could be those biopharmaceuticals that can exert their desired pharmacological effect with minimum side effects to enhance patient compliance.

Macrophage Colony-Stimulating Factor

MCSF (also known as CSF-1), is a homodimeric growth factor that specifically required to regulate the survival, proliferation, motility, differentiation and functions of cells of monocyte/macrophage lineage.[155, 156] MCSF is a pleiotropic cytokine that mediates a broad range of biological activities. In the hematopoietic system, it is required to activate the precursor monocytoid cells to become better phagocytic cells. MCSF primarily stimulates the proliferation, differentiation, growth and survival of macrophages and resident macrophages of local tissue such as kupffer cells in liver, microglial cells in bone, mesangial cells in the kidney, osteoclasts in bone, etc and affects immunological activities of mature macrophages including antigen presenting, phagocytosis and antitumor cytotoxicity.[157-160] It also helps the generation of two subsets of dendritic cells. In the skin, the langerhans cells are stimulated, while in the blood and lymph nodes, plasmacytoid dendritic cells are produced.[158] It had been found that MCSF enhances both class I and class II MHC-restricted antigen presentation pathways in dendritic cells. In addition, it was found that MCSF increases intracellular processing events of phagocytosized antigen in dendritic cells.[161] MCSF also indirectly modulates the hematopoiesis and immunological effects by influencing the expression of cytokines including GMCSF, IL-8, IL-1, TNFs, INF- γ, and GCSF. [157, 162] In non- hematopoietic system, MCSF is an important regulator for the proliferation and differentiation of osteocytes , trophoblasts, and breast epithelial cells.[157, 163] In addition, this cytokine is associated with some pathological processes and diseases, such as arteriosclerosis, gynecologic malignancies, hepatocellular carcinoma, breast cancer and chronic renal failure. All suggest that the biological activities of MCSF are diverse and sophisticated.[157]

Initially, the human form of MCSF was isolated from urine.[158] Later it was discovered that a variety of cell types including endothelial cells, stroma cells, fibroblasts, [159] macrophages,[164] neurons, astrocytes and microglia cells [165, 166] have been found to secrete MCSF. Blood monocytes also secrete MCSF *in vitro* when they adhere to plastic dishes *in vitro* or in response to cytokines such as TNF-α, IFN- γ, or GMCSF.[159] Some tumor cells are also known to express isoforms of MCSF and the expression of these isoforms, especially those located to the cytoplasma and nucleus, was reported to be related to the prognosis and metastasis of tumors. [157] The human MCSF gene is located in the short arm of chromosome 1, band p13-p21 and its 4.0 kb cytoplasmic mRNA encodes an 85 kDa homodimeric bioactive MCSF protein.[24, 159] Mature MCSF is a glycoprotein containing three potential N-linked glycosylation sites with a molecular mass of 45-90 KDa.[14] MCSF is encoded by a single gene, but can exist in different forms due to alternative splicing.[157] Two forms of MCSF have been isolated and characterized so far from biological fluids and cell cultures: monocytic MCSF which has a molecular weight of 40-70 kDa (short form) and urinary MCSF which has a molecular weight of

70-90 kDa (long form).[24] The biologically active form of MCSF is a homodimeric glycoprotein exists as integral cell surface proteins or may be released from their producer cell by proteolytic cleavage to yield the soluble cytokine.[14] MCSF exerts its pleiotropic effects via a high-affinity transmembrane type III tyrosine kinase receptor (MCSF receptor), which has been identified as the product of a proto-oncogene c-fms.[156, 164] The MCSF receptor is a single chain, heavily glycosylated polypeptide of molecular mass 150 KDa and is expressed on the surface of different hematopoietic and non-hematopoietic target cells.[14, 159, 163, 165]

MCSF was isolated and purified from human urine (hMCSF) and has been used clinically in patients with granulocytopenia associated with anticancer chemotherapy and to promote increases in granulocytes after bone marrow transplantation.[162] The recombinant MCSF (called Lanimostim/MacroTac) is also used clinically in bone marrow transplantation patients, whose innate immune system has not been fully restored, and consequently suffer from recurrent fungal and bacterial infections due to the lack of myeloid cells. Infused MCSF also activates these monocytoid cells to become better phagocytic cells, thereby clearing the microbes by directly engulfing the pathogens. One of the major toxicity that limits the therapeutic use of this cytokine is thrombocytopenia due to destruction of the platelets, which have the same approximate size as the microbes, by the MCSF-activated monocytes/macrophages mediated phagocytosis. The MCSF-induced thrombocytopenia is reversible, following cessation of the treatment.[158] To minimize the risk of MCSF-induced thrombocytopenia and other dark sides of MCSF, it is hoped that the next generation of MCSF could be those biopharmaceuticals which can exert their desired pharmacological effect with minimum side effects.

Thrombopoietin

Platelets are responsible for primary hemostasis and are produced by the cytoplasmic fragmentation of bone marrow megakaryocytes.[167] The existence of TPO, a specific humoral regulator of platelet production, was first proposed over 50 years ago. That such a substance existed was further supported by the subsequent demonstration that plasma, serum and urine from thrombocytopenic animals could be used to stimulate platelet production in other animal models. The development of the first *in vitro* assays for human megakaryocyte progenitor cells in 1979 allowed further definition of the regulators of human megakaryocytopoiesis and thrombopoiesis. However, it was not until 1994, when the ligand for the HGF receptor c-Mpl was cloned, and found to have profound effects on both megakaryocytopoiesis *in vitro* and platelet production *in vivo*, that a single substance was finally demonstrated, which significantly stimulated these processes.[168]

TPO or c-Mpl ligand (also known as megakaryocyte growth and development factor (MGDF)) [169] is a physiologic regulator of platelet and megakaryocytic production, acting synergistically on thrombopoiesis with the growth factors IL-11, stem cell factor, IL-3, IL-6 and GMCSF.[170] Several studies had shown that TPO not only influences the megakaryocyte/ platelet lineage but also plays a significant role in maintaining stem cells and promoting other hematopoietic lineages, most likely as a result of its ability to inhibit apoptosis in these cells.[170-172] It had been also investigated that TPO might stimulate vascular endothelial growth factor (VEGF) production. It had been demonstrated that TPO causes a marked increase of VEGF release in cell lines that express the TPO receptor c-Mpl. Furthermore, it had been reported that *in vitro*, production of VEGF by hematopoietic progenitor cells is specifically associated with TPO-induced differentiation, and not with the differentiating effects of other similarly acting cytokines.[173] In addition,

accumulating evidence indicates that TPO has been shown to stimulate *ex vivo* platelet aggregation and a granule secretion in the presence of platelet agonists. This suggests that TPO would promote the restoration of radiation-induced endothelium damage through the stimulation of platelet functions. On the other hand, TPO might act directly on endothelium, as expression of its receptor c-Mpl has been reported on endothelial cells.[174] Taken together, these facts indicate that TPO is one of the most important HGFs yet identified and exerts diverse biological actions beyond megakaryocytopoiesis.

Human TPO is a 60 -70 kDa, 332 amino acid residue glycosylated protein comprises an amino-terminal domain including 4 cysteine residues and a carboxyl-terminal domain including 6 potential N-glycosylation sites. [175, 176] It is primarily produced by liver (in hepatocytes) and, to a lesser degree, kidney (in convoluted tubular cells), bone marrow (in stromal cells) and spleen. [177-179] TPO gene expression has also been detected in skeletal muscle, ovary, testis and fetal lung. Moreover, this cytokine is produced also in the central nervous system where its generation appears to be locally restricted.[179] TPO mRNA has been detected only in the corpora amygdala and the hippocampus, but not in other areas of the central nervous system.[180] The regulation of TPO production is not yet fully elucidated but current evidence suggests that TPO production is constant, with the circulating TPO level being inversely related to the amount of its receptor (c-Mpl) available on platelets, megakaryocytes and their precursors to bind, internalize and metabolize TPO.[168] The biological actions of TPO are initiated by specific binding to cell surface receptors expressed on target cells. The TPO receptor, c-Mpl, is transmembrane receptor that belongs to a member of a cytokine type I receptor superfamily.[172] It is found on megakaryocyte precursor cells, megakaryocytes, platelets, stem cells and on bone marrow progenitor cells. [181,182] Recent studies investigating the role of TPO in neuronal function demonstrated the expression of TPO receptor in neurons, astrocytes and neuroblastoma-derived cells.[172]

The activation of signal transduction from the c-Mpl receptor following TPO binding is thought to be initiated by ligand dependent receptor homodimerization. [183] Members of the JAK family as well as other intracellular tyrosine kinases are subsequently activated, resulting in phosphorylation of a number of signaling molecules as well as the receptor itself. Several signal transduction pathways are mobilized upon TPO stimulation of c-Mpl-expressing cells, including the STATs, PI 3- K and MAPK cascades as shown in Figure 2. Such biochemical changes signal the biological outcomes that typify TPO action, including cell proliferation, maturation or survival. [172, 181, 183,184]

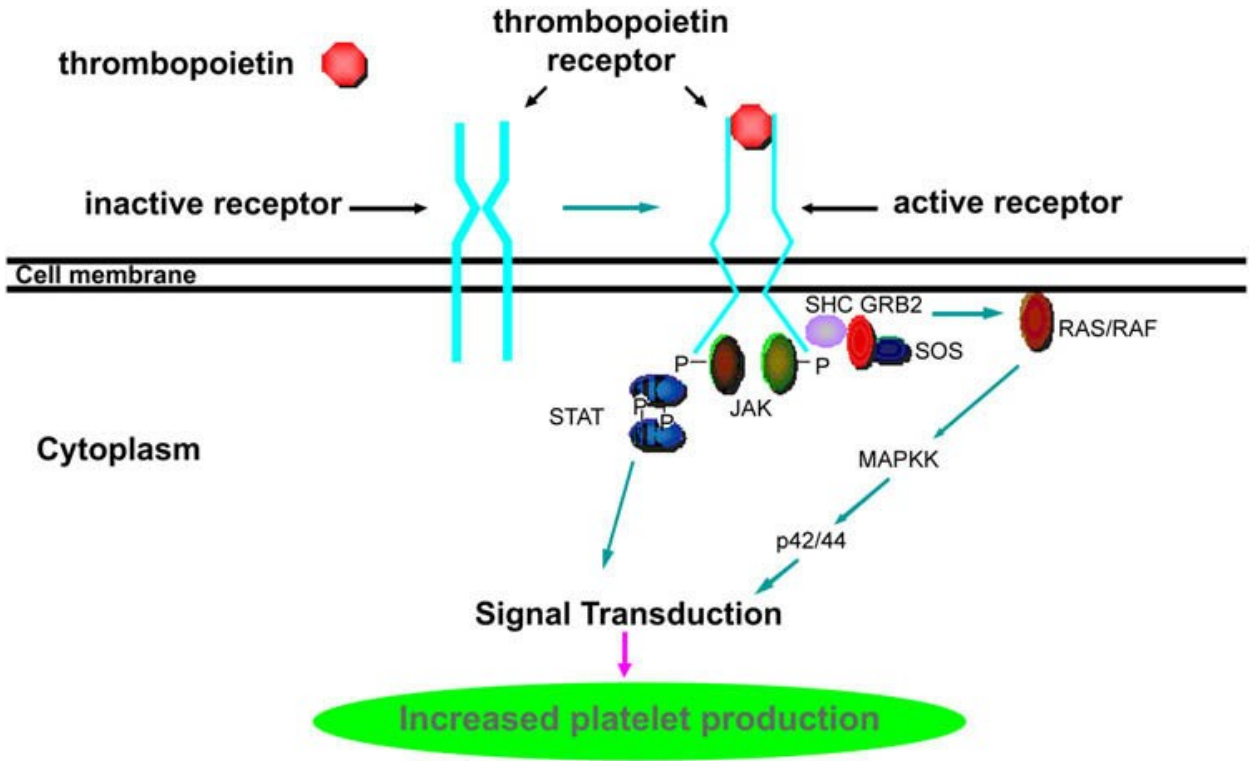

Figure 1: Mechanism of action of TPO[181]

After nearly a 50-year search for a key regulator of platelet production, in 1994, five different groups reported on identification and cloning of the cDNA for the c-Mpl ligand, TPO. Since the time of its cloning, TPO rapidly moved from laboratory to clinic in 2 years.[185] Recombinant human TPO, which is produced by recombinant DNA technology using CHO cells, is a potential therapeutic glycoprotein for amelioration of thrombocytopenia caused by chemotherapy, irradiation, bone marrow transplantation,[186] inflammatory states, neoplasia, [176] liver disorders such as complications associated with liver cirrhosis [182, 187] and destruction of platelets by immunological processes.[188] Two forms of recombinant human TPO were developed for clinical evaluation: the full-length glycosylated molecule known as recombinant human TPO (Genentech, Inc, San Francisco, CA) and the truncated version bound to polyethylene glycol known as PEGylated recombinant human MGDF (Amgen Inc, Thousand Oaks, CA).[185] Both recombinant forms showed potent platelet stimulatory activity and excellent clinical tolerance in the initial phase I clinical trials. Subsequent clinical development of such first generation recombinant forms of TPO, however, has been slower, partly because of its unique biology and nature of response and partly because of difficulties associated with a protein-based drug, such as limited administration methods and the unfortunate development of neutralizing antibodies in patients receiving PEGylated recombinant human MGDF.[181, 185, 189] In looking for solutions to such problems, more recently, two TPO mimics, which are second-generation thrombopoietic growth factors, having no sequence homology with natural TPO, were approved by the FDA for use in patients with severe refractory immune thrombocytopenic purpura. [181, 190] These drugs including romiplostin (a 60-kDa TPO antibody mimetic) and eltrombopag (a TPO non-peptide mimetic) [181] act to stimulate platelet production on a large scale, allowing marrow compensation for ongoing antibody-mediated platelet destruction and/or inhibition of megakaryocyte development in patients, normalizing the platelet counts in the majority of patients given an adequate therapeutic dose.[190] In addition to second generation thrombopoietic growth factors, pleiotropic cytokines have been shown

to have a generally modest effect on thrombocytopenia. In particular, IL-11 has successfully been shown to reduce the incidence of severe thrombocytopenia in patients receiving intensive chemotherapy, and has been approved by FDA for the treatment of severe thrombocytopenia in patients receiving myelosuppressive therapy. However, side effects are common and particularly limiting in patients with liver disease. [181, 191]

Interleukin-3

Hematopoiesis is a complex process of cell proliferation and differentiation that is regulated by a variety of CSFs (as discussed above). A number of the interleukin families of cytokines are also known to influence hematopoiesis. IL-3 is one of the major hematopoietic cytokines that play an important role in hematopoiesis associated with inflammation or immune responses.[192] IL-3, also known as multi-lineage CSF, is expressed by mitogen or antigen activated T-lymphocytes,[193,194] natural killer cells,[194] keratinocytes, endothelial cells, monocytes/macrophages, mast cells,[195] neurons and microglial cells.[166, 193] IL-3 was originally identified by its ability to induce the synthesis of 20 - α - steroid dehydrogenase in splenic lymphocytes of nude mice. It is a potent growth promoting cytokine that has a very broad spectrum of activities in regulating biological responses such as cell proliferation, survival, growth and differentiation.[194, 195] For example, it plays very important roles in the proliferation and differentiation of a broad range of hematopoietic progenitor cells into erythrocytes, monocytes, macrophages, megakaryocytes, mast cells, basophils, neutrophils, eosinophils, dendritic cells,[195, 196, 197] microglial cells and placental cells.[198] In addition to its effects on the development of different hematopoietic cells, IL-3 can also enhance antigen presentation for T cell-dependent responses, augment macrophage cytotoxicity and adhesion, promote the secretory function of eosinophils and basophils, participate in inflammation by inducing expression of adhesion molecules on human endothelial cells,[196] augment the activity of natural cytotoxic cells,[199] specifically induces the production of enzymes involved in cellular metabolism, differentiation, and DNA/RNA metabolism [200] and promote glucose transport into cells.[201]

IL-3 exerts its biological activities through binding to a high-affinity, specific cell surface receptors that are located on bone marrow progenitors, macrophages, mast cells, eosinophils, megakaryocytes, basophils and various myeloid leukemic cells.[200] The high affinity receptors for IL-3, IL-5 and GMCSF are composed of two subunits, α- and ß-subunits, both of which are members of the class I cytokine receptor family. The α- subunits are specific for each cytokine and bind their specific ligands with low affinity, whereas the ß- subunit is required for high affinity binding as well as signaling by all three receptors.[192, 202, 203] IL-3 is known to activate at least three signaling pathways: the Jak/STAT, the Ras/Raf/MAP kinase, and the PI 3-K /protein kinase B (PKB) pathway.[195] It is distinct among the HGFs in having the capacity to stimulate progenitor cell renewal. It is used in combination with other hematopoietic factors to stimulate blood cell regeneration after bone marrow engraftment, chemotherapy or irradiation.[200] After the cloning of the murine IL-3 gene, the human IL-3 gene was first cloned and identified in 1988; its genomic DNA has a length of approximately 2.2 kb and contains five exons. It is located on chromosome 5 at segment 5q23-31, clustered with GMCSF, IL-5, IL-4, IL-9 and IL-13. [195, 200] This gene shown to code for a protein of 152 amino acids long, and the mature human IL-3 protein is a 15-17 kDa glycoprotein containing 133 amino acids with two conserved asparagines for potential N-linked glycosylation sites at positions 15 and 70 and contains a single disulfide bond (Cys16/84).[195, 200, 204] Recombinant human IL-3 has been synthesized chemically and in several expression systems,

including bacteria, streptomyces, CHO cells, baculovirus expression vector system [195, 200] and the yeast, *Pichia pastoris.*[195] Among these expression systems, the baculovirus expression vector system and expression in *Pichia pastoris,* have become popular in recent years. The baculovirus expression vector system has the major advantage of producing high yields of recombinant proteins in eukaryotic cells. It was exploited to express mouse IL-3 and human insulin-like growth factor - IL-3 chimeras.[200] The *Pichia pastoris* expression system is also increasingly recognized as an ideal system for the expression of active recombinant proteins due to its low cost, ease of genetic manipulation and growth to a high cell density. Additionally, it does not produce pyrogenic endotoxins as the bacterium does. In fact, many pharmaceutically important proteins have successfully been produced using this system for clinical applications.[195] Recombinant IL-3 has been widely used in clinical practice, mainly for the purpose of targeting the phases of leukocytopenia and bone marrow suppression during the treatment of leukemia. IL-3 was also used as a drug in therapy of patients with deficiency of the bone marrow function caused by progressive tumors as well as in the treatment of lung cancer, aplastic anemia, myelodysplasia and thrombocytopenia.[195, 204, 205]

SUMMARY

HGFs are glycoproteins with diverse roles to play as therapeutic agents for different pathological conditions. Originally, their use was limited to hematopoietic disorders but, at present their non-hematopoietic functions are discovered. These novel effects of HGFs are extensively studied by making use of the recombinant forms of different HGFs. These studies focused on investigating the effect of recombinant HGFs such as EPO, GMCSF, GCSF, MCSF, TPO and IL-3 on diseases of brain, heart, kidney and other organs. The studies were conducted on animal models and the results obtained are encouraging and with a further all round studies, recombinant HGFs might be a promising biopharmaceuticals for their intended novel therapeutic applications in the treatment of both hematopoietic and non-hematopoietic associated diseases. Generally, the field of HGFs is dynamic. New HGFs are being discovered. The indications for commercially available HGFs are expanding. Clinical experience will also lead to the development of more convenient delivery systems and formulations that are used to minimize their serious adverse effects associated especially with immunogenic reactions and to enhance their desired pharmacological efficacy to establish sustainable clinical benefits from them.

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