Hematopoietic Effects and Clinical Application of Granulopoietic Growth Factors

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INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF)\(^1\) regulates the production of neutrophil granulocytes, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF)\(^2\) stimulates multiple lineages of hematopoiesis including neutrophopoiesis and eosinopoiesis. Both these hematopoietic growth factors (HGF) have complex and yet not fully characterised effects on granulocyte function. G-CSF and GM-CSF are used to reduce chemotherapy related neutropenia and to mobilise blood progenitor cells (BPC) for autologous transplantation\(^3\). Recently, G-CSF has also been administered to healthy donors for collection of BPC and granulocytes, respectively\(^4,5\).

The present thesis focuses on G-CSF - its effect on neutrophil function, use in remission induction chemotherapy for ALL and for mobilisation of blood progenitor cells in normal donors. In addition, the effects of GM-CSF on the regenerating granulopoiesis after ABMT is studied and discussed.

ABBREVIATIONS: ABMT=autologous bone marrow transplantation; ABPCT=autologous blood progenitor cell transplantation; ALL=acute lymphoblastic leukaemia; AML=acute myeloblastic leukaemia; ANC=absolute neutrophil count; BFU-E=burst-forming unit-erythrocyte; BPC=blood progenitor cell; BPI=bactericidal permeability-increasing protein; CD=cluster of differentiation; CFU-GM=colony-forming unit-granulocyte/monocyte; ECP=eosinophil cationic protein; ELISA=enzyme-linked immunosorbent assay; EPO=erythropoietin; f-MLP=formyl-methionyl-leucyl-phenylalanin; G-CSF=granulocyte colony-stimulating factor; GM-CSF= granulocyte-macrophage colony-stimulating factor; GVHD=graft versus host disease; HGF=hematopoietic growth factor; HNL=human neutrophil lipocalin; ICAM=intercellular adhesion molecule; IL=interleukin; LF=lactoferrin; LTC-IC=long-term culture initiating cell; M-CSF=macrophage colony stimulating factor; mab=monoclonal antibody; MPO=myeloperoxidase; P=plasma; PMA=Phorbol myristate acetate; S=serum; SCF=stem cell factor; TNF=tumour necrosis factor; TPO=trombopoietin.
HEMATOPOIESIS

Adult hematopoiesis (Fig 1) derives from a small pool of pluripotent hematopoietic stem cells (PHSC) residing mainly in the bone marrow. PHSC are cells with extensive proliferative potential and with capacity to self-renewal and to differentiate into all hematopoietic lineages. There is no established assay for the quantification of these cells.

At a given time, the vast majority of PHSC are non-dividing, whereas a small proportion is proliferating to give rise to differentiated progenitor cells. Lineage-committed progenitor cells, defined by the ability to form colonies in semisolid culture assays (CFU-GM, BFU-E etc), matures into morphologically recognisable precursor cells (myeloblasts, erythroblasts, megakaryoblasts etc). Following terminal differentiation, mature blood cells are released into the circulation.

The hematopoiesis is regulated by its interaction with stromal cells, molecular components of the extracellular matrix and with HGF. The role of inhibitors of hematopoiesis is less well defined than that of stimulators. HGFs are soluble glycoproteins which stimulate the production, survival and activity of hematopoietic cells. HGF act either locally in the bone marrow microenvironment or systemically as circulating "hormones". Other common features are pleiotropy, overlapping activities and the ability to act both directly on hematopoietic target cells and indirectly by inducing the production of other cytokines.

More than 20 HGF have been biochemically characterised and genetically cloned. HGF presently used in clinical practice or trials include EPO, TPO, G-CSF, GM-CSF, M-CSF, SCF, IL-2, IL-3, IL-6 and IL-11.

BLOOD PROGENITOR CELLS

Hematopoietic progenitor cells, including primitive progenitors, are present in the circulation at a low frequency. The proportion of progenitor cells (CD34+) among mononuclear cells has been estimated to 0.1% in blood and 1-2% in the marrow. Recent in vitro studies indicate that the proliferative capacity of blood CD34+ cells is similar to that of bone marrow CD34+ cells. Steady state blood mononuclear cells are capable of restoring myeloablated hematopoiesis in experimental animals and in humans.

NEUTROPOIESIS

Neutrophil production

An adult of average size has to produce approximately $1 \times 10^{11}$ granulocytes per day simply to replace normal losses. This production may be increased at least 10-fold under conditions of stress such as acute infections. Of the total pool of neutrophil granulocytes, only 5-10% are found in the circulation.
The earliest *morphologically* recognisable neutropoietic cell is the myeloblast. Differentiation from myeloblast to mature granulocyte includes four to eight cell divisions and occurs normally over 7-10(14) days. Half of this time is spent in the post-mitotic compartment (neutrophil storage pool) as metamyelocytes, band and segmented neutrophils, respectively. Mature neutrophil granulocytes egress from the bone marrow into the circulation through the endothelial lining of marrow sinusoids into the blood stream. In the circulation, neutrophils normally spend 6-8 hours ($t_{1/2}$) before being eliminated in the liver and spleen.

**Neutrophil function.**

Neutrophil granulocytes play a key role in the first line defense against bacterial and fungal infections. Neutrophils, including immature forms, may be released from the marrow in response to cytokines or other mediators of inflammation. Further steps in the neutrophil inflammatory response are adhesion, migration, phagocytosis, degranulation and microbial killing.

Adhesion of granulocytes to endothelial cells is mediated by the induction and activation of adhesion molecules on endothelial cells and on granulocytes. Neutrophil granulocytes are able to adhere to the endothelial cell adhesion molecules P-selectin, E-selectin and ICAM-1. Adhesion to ICAM-1 is mediated through the adhesion molecules LFA-1 (CD11a/CD11b) and Mac-1 (CD11b/CD18) on granulocytes, adhesion to P-selectin by PSGL-1 (P-selectin glycoprotein ligand 1) and adhesion to E-selectin by sialyl-LewisX. Granulocyte adhesion to ICAM-1 is necessary for the subsequent transendothelial migration of granulocytes.

Once in the tissues, neutrophils are attracted to the site of inflammation by chemotactic factors, which are generated by the microorganisms or by the inflammatory reaction itself. Activated neutrophils adhere to opsonised microorganisms by surface receptors for complement (CR1, CR3) and the constant region of IgG (FcγRI-III), respectively. Following phagocytosis, microorganisms are finally killed by the cytotoxic reactive oxygen metabolites of respiratory burst and by the action of certain granule proteins such as MPO, lysozyme, LF, BPI and defensins (oxygen-independent microbiocidal killing).

**Neutrophil granule proteins.**

Neutrophil granulocytes contain primary (azurophilic) and secondary (specific) granules. Primary granules are formed at the myeloblast-promyelocyte stage of differentiation and contain MPO, lysozyme, cathepsin G, elastase, defensins as well as other antimicrobial agents. Secondary granules are formed at the myelocyte-metamyelocyte stage and contain a number of different proteins including LF, specific collagenase and lysozyme. Gelatinase-containing granules are considered as a subpopulation of secondary granules. Secondary granules contain in their membrane several...
adhesion- and chemotactic receptors, which are mobilised to the cell surface upon neutrophil activation 40.

MPO catalyses the reaction of peroxide with chloride to form hypochlorous acid, which is highly toxic for a variety of microorganisms 41. LF inhibits the growth of ingested microorganisms by sequestering free iron 42 and has been implied a role as inhibitor of granulopoiesis, although the latter remains controversial 43.

HNL is a recently characterised N-glycosylated protein with a molecular weight of 45 kDa (unreduced). It was isolated from neutrophil secondary granules 44. HNL is identical to the previously described neutrophil gelatinase-associated lipocalin, and is in part covalently associated with gelatinase 44,45. HNL and LF have a different subcellular distribution, and may be released differently upon neutrophil activation 46.

**Serum/plasma levels of neutrophil granule proteins.**

Neutrophil granule proteins are secreted upon activation and phagocytosis, or may be passively released due to cell damage or death 47. Activated neutrophils secrete their granules into the phagosome as well as into the extracellular environment. Sensitive immunoassays have been developed for measuring MPO, LF and HNL, respectively, in cells as well as in various body fluids 48,49. Circulating levels of MPO and LF reflect the neutropoietic activity of bone marrow 48. Blood measurements of these granule proteins may be used to monitor bone marrow regeneration after chemotherapy including (A)BMT 50,51.

Blood levels of neutrophil granule proteins are not only influenced by the number and turnover of neutrophils, but also by their *in vivo* secretory activity 52. Thus, highly elevated serum/plasma levels of LF and HNL are observed in conditions with neutrophil activation such as acute infections 53,54. S-HNL has been shown useful in discriminating between bacterial and viral infections 54.

Serum levels of granule proteins are the result of plasma levels plus what has been secreted from the neutrophils *ex vivo* in the test tube, partly as a consequence of factors produced during coagulation 55. This *ex vivo* secretion is more pronounced when the neutrophils have been activated *in vivo*.

**GRANULOPOIETIC GROWTH FACTORS**

G-CSF and GM-CSF are the two major granulopoietic growth factors. GM-CSF stimulates proliferation and differentiation of hematopoietic cells mainly of the granulo-monocytopoietic and eosinophilic lineages 56. Apart from its effects on progenitor and precursor cells, GM-CSF is a potent activator of mature neutrophils, eosinophils and monocytes 2,57,58. Its physiological role is unclear, although studies of the GM-CSF knock-out mouse suggest that it is important for macrophage function 59. Therapeutically, recombinant GM-CSF
is mainly used to stimulate neutropliosis in myelodysplastic syndrome, following chemotherapy and after bone marrow transplantation. Its clinical application has been hampered by dose-dependent side effects such as fever. The immunomodulating effects of GM-CSF are presently being explored in clinical trials.

This thesis focuses on G-CSF, which is therefore discussed below in more detail.

G-CSF - PRECLINICAL CHARACTERISTICS

Identification
In the mid-1960s cell culture assays for hematopoietic progenitor cells were developed. From studies using these assays it became clear that survival, proliferation and differentiation of hematopoietic cells are dependent on the presence of humoral factors - named colony-stimulating-factors (CSFs). Further purification of these factors turned out to be extremely difficult. Thus, the different CSFs were long defined as biological activities depending on the assay system used.

In 1980, G-CSF was identified by the capacity of postendotoxin treated mice sera to induce differentiation of the murine myelo-monocytoid cell line WEHI-3B(D+)66. Further studies showed that this factor selectively stimulated the formation of granulocytic colonies from normal murine hematopoietic progenitor cells, and it was after further purification named "G-CSF". In 1986, human G-CSF was ultimately purified to homogeneity from CSF-producing tumour cell lines68,69. Subsequently, a cDNA encoding human G-CSF was isolated from the bladder carcinoma cell line 5637 and expressed in E coli by Souza et al70. Independently, a cDNA for G-CSF was isolated from the squamous carcinoma cell line CHU-2 and expressed in Chinese hamster ovary cells by Nagata et al71.

During the period 1984-1987, the genes encoding GM-CSF, M-CSF, EPO and IL-3, respectively, were also cloned72-74. These breakthroughs enabled the large scale production of HGF for in vivo studies. Already in 1987 the first clinical trial with recombinant G-CSF was reported75.

Structure
Native human G-CSF is an o-glykosylated 20 kD polypeptide consisting of 174 amino acids and a carbohydrate chain which accounts for ≈4% of the molecular weight70,76. It is encoded by a single gene located on chromosome 17q11-22. Before secretion, the G-CSF molecule is glykosylated. Highly purified human G-CSF and recombinant G-CSF have identical biological activity77,78.

The chinese hamster ovary derived G-CSF (rHuG-CSF; lenograstim) is structurally identical with the naturally occurring G-CSF79, whereas the E-coli derived recombinant form (r-metHuG-CSF; filgrastim) has an extra amino
acid at the N-terminal end and is non-glykosylated. The function of the glycosylated part of the molecule is unknown, although it has been hypothesised that the sugar chain confers a greater resistance to inactivation and enzymatic degradation.

### Cellular production.

G-CSF is produced by monocytes-macrophages, fibroblasts and endothelial cells. Small amounts of G-CSF may also be produced by the neutrophils themselves. In vitro, bacterial endotoxins, IL-1, and TNFα may induce the synthesis of G-CSF by fibroblasts and endothelial cells. G-CSF is also produced by monocytes-macrophages after stimulation by T-cell derived cytokines, such as IL-3 and GM-CSF. Malignant cells producing G-CSF are responsible for the paraneoplastic leukemoid reaction sometimes seen in association with certain tumours such as malignant melanoma, hepatoma and sarcoma.

### Receptor

Like other cytokines, G-CSF exerts its actions by binding to high-affinate receptors. The G-CSF receptor (G-CSF-R) is a single 813 amino acid polypeptide and consists of a cytoplasmatic, a transmembrane and an extracellular domain. Recent findings indicate that the cytoplasmic region proximal to the membrane transduces proliferative and survival signals, whereas the distal C-terminal region transduces maturation signals and suppresses proliferative signals.

G-CSF-receptors are present in the neutrophil lineage on cells from the myeloblast to the segmented neutrophil granulocytes. Mature neutrophils and their precursors possess a small number of these receptors. AML clonogenic cells commonly express low levels of G-CSF receptors. However, the presence of these receptors per se does not predict that the AML cells proliferate in response to G-CSF.

### Circulating levels

In healthy individuals, serum levels of endogenous G-CSF are usually low, i.e. below the detection limit of most currently used methods. High blood levels of G-CSF have been shown in the early phase of bacterial infections as well as in various conditions associated with severe neutropenia. Decreasing S-G-CSF precedes or parallels neutrophil recovery after stem cell transplantation. High levels of S-G-CSF during neutropenia may be due to increased production, decreased clearance or both.

Notably, serum levels during severe infections are comparable with what is observed after treatment with standard doses of recombinant G-CSF.
Physiological role.
Recent experiments with the G-CSF gene knock-out mice (G-CSF-/-) suggest that G-CSF is of key importance both in maintaining steady-state hematopoiesis and in the hematopoietic response to environmental stress. Thus, G-CSF-/—mice have a severe chronic neutropenia, a reduction in the mobilisable neutrophil reserve and a reduced marrow granulopoiesis. In addition, G-CSF-/—mice show a reduced ability to control infections, suggesting that G-CSF is necessary for the normal emergency granulopoietic response.

In vitro effects
G-CSF stimulates proliferation, differentiation and survival of neutrophil progenitor and precursor cells. G-CSF also enhances mature neutrophil survival, adherence and primes the respiratory burst. G-CSF stimulates the growth of committed myeloid progenitors (CD34+CD33-), whereas cultures enriched for primitive progenitors (CD34+CD33-) show no or minimal response to stimulation with G-CSF alone. However, G-CSF primes primitive cells to increased response to “early acting cytokines” such as IL-1, IL-3, IL-6 and SCF.

Pharmacological effects
Administration of G-CSF induces an acute margination of neutrophils, as manifested by a transient drop in ANC between 15-30 minutes post s.c. injection. The initial drop is followed by a sharp increase in ANC starting 2-3 hours and reaching peak levels at about 12 hours post injection. This early increase may be due to mobilisation of neutrophils from the bone marrow storage pool. Following the continued administration of G-CSF, there is a dose-dependent progressive neutrophilia plateauing after 7-8 days. After discontinuation of G-CSF, ANC return to pretreatment values within 3-4 days. More detailed information about the effects of G-CSF on the neutropoiesis comes from cell kinetic studies in humans and mice, using tritiated thymidine (3H) TdR and autoradiography. Thus, G-CSF was shown to increase the proportion and proliferation rate of neutrophilic precursors, and to reduce the myelocyte-to-blood transit time to 1-2 days (normal 4-5 days). These results further suggest that G-CSF excerts its proliferative effect mainly on the stage of myeloblast-promyelocyte, whereas effects on more primitive progenitors are limited. G-CSF has no apparent impact on the circulation half-life of neutrophils.

G-CSF causes a dose-dependent increase in the monocyte count which is modest relative to the neutrophil response. High doses (≥10 μg/kg/d) of G-CSF may be associated with a transient decrease in platelet count. The effect of G-CSF on progenitor cell mobilisation is discussed below.
Effects on neutrophil function
Neutrophils collected from patients or healthy volunteers receiving single or repeated doses of G-CSF show increased adherence\(^{106}\), enhanced phagocytosis\(^{122}\) and increased superoxide anion (O\(_2^-\)) production in response to f-MLP\(^{123}\). G-CSF increases the surface expression of adhesion molecules\(^{124-126}\) and Fc receptors for IgG\(^{114,127}\). Results with respect to effects on neutrophil chemotaxis are inconsistent\(^{128-130}\).
Some of the observed effects on mature neutrophils might be indirect or reflect the increasing immaturity of the neutrophil population following G-CSF\(^{114,130}\).

G-CSF - CLINICAL APPLICATIONS

G-CSF is presently used in a number of different clinical settings, ranging from generally accepted/formally approved (i.e. congenital neutropenia, post chemotherapy, post ABMT, mobilisation of PBPC for autologous transplantation) to clearly experimental indications (infections in non-neutropenic patients) (Table I).

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*for granulocyte transfusion ** no definite clinical results available
In line with the present thesis, the following discussion will be focused on the use of G-CSF to reduce infectious morbidity after chemotherapy induced neutropenia, and for mobilisation of blood progenitor cells.

**CHEMOTHERAPY INDUCED NEUTROPENIA**

**Infections during neutropenia**
Chemotherapy induced neutropenia is frequently complicated by infections or fever suspected of infection \(^{147}\). The risk of infection correlates with the depth and duration of neutropenia \(^{25}\). Apart from neutropenia (ANC<0.5x10^9/l), other factors related to the underlying disease or its treatment may increase the incidence of infections. These include mucositis, immunosuppression, defect granulocyte function and the use of indwelling intravenous catheters. Prophylactic antibiotic treatment may decrease the risk of gram negative infections in neutropenic patients, although its role is still controversial \(^{148}\).

Patients presenting with "febrile neutropenia" (fever and ANC<0.5x10^9/l) are usually readmitted for treatment with broad-spectrum intravenous antibiotics. This therapy is generally continued until resolution of clinical symptoms and neutropenia \(^{149}\). The cause of fever cannot be identified in 60-70% patients presenting with febrile neutropenia \(^{150}\). Both infections and prolonged neutropenia *per se* may also lead to delays and dose-reduction of chemotherapy, thereby potentially compromising treatment efficacy.

**G-CSF after standard dose chemotherapy in solid tumours**
Treatment with G-CSF after myelosuppressive chemotherapy shortens the period of neutropenia and time to neutrophil recovery \(^{75}\). G-CSF may thus reduce delays in chemotherapy and prevent dose reductions \(^{151}\). No advantage of G-CSF treatment in terms of tumour response or survival has yet been shown \(^{133}\). Several ongoing studies explore the use of G-CSF in the context of dose escalation.

Three randomised trials in patients with solid tumours have demonstrated that the administration of G-CSF after chemotherapy may reduce the incidence of febrile neutropenia by approximately 50%. Patients receiving G-CSF required fewer days in hospital due to infections and had less days with antibiotics. It is not clear whether the number of days with fever (i.e. irrespective of ANC) was reduced. Importantly, in all these trials the incidence of febrile neutropenia in the control group was as high as >40% \(^{131,152,153}\).

G-CSF seems to have no impact on infectious morbidity after less myelosuppressive chemotherapy \(^{133}\).

**G-CSF in acute leukemia.**
In AML, the use of growth factors has been restricted due to concern about the possible stimulation of leukaemia cells by such treatment \(^{154}\). However, recent
studies show that patients receiving G-CSF after induction chemotherapy for AML have a similar or even better complete remission rate compared with controls 155-158. Still, no impact on treatment-related mortality or long-term survival has been demonstrated. G-CSF reduces chemotherapy related neutropenia, but an impact on infectious morbidity (i.e. incidence of infections, fever) has not been consistently shown 156,158. There are yet few data regarding the possible value of granulopoietic growth factors during induction treatment of ALL. In a recently published study, the administration of G-CSF during the second phase of remission induction chemotherapy had no clear impact on infectious morbidity 159.

G-CSF after stem cell transplantation
Treatment with G-CSF after ABMT shortens time to neutrophil engraftment (ANC>O.1x10^9/l) by 3-4 days, whereas time to neutrophil recovery (ANC>0.5x10^9/l) is reduced by with approximately one week 134,160. Doses of G-CSF above 5 μg/kg/d does not seem to confer any advantage in terms of neutrophil recovery 161. Uncontrolled trials suggest that the start of G-CSF treatment may be delayed until six days post ABMT 162. Results from two phase III trials indicate that treatment with G-CSF after ABMT reduces neither the incidence of infections nor the number of days with fever 134,163. The lack of impact of G-CSF on infections might reflect the fact that patients still are profoundly neutropenic during the first week post ABMT 137. Nevertheless, patients receiving G-CSF after ABMT have fewer days on antibiotics and total parenteral nutrition and require shorter time in hospital post transplant. This may reflect an impact of G-CSF on the duration or severity of infections and/or the fact that clinical decisions are influenced by knowledge of the neutrophil count. G-CSF has a modest effect on neutrophil recovery (ANC>O.5x10^9/l) after autologous blood progenitor cell transplantation135. An impact on infectious morbidity has not been shown. Treatment with G-CSF after allogeneic BMT hastens neutrophil recovery without adversely affecting the incidence or severity of acute GVHD 134,136. Reduction in infectious morbidity has not been clearly shown.

BLOOD PROGENITOR CELLS - MOBILISATION AND TRANSPLANTATION

Mobilisation of BPC
Mobilisation of hematopoietic progenitor cells may be induced by a number of different agents, including endotoxin, dextran, chemotherapy and various HGFs (reviewed) 164,165. Depending on the stimuli used, mobilisation occurs within 30 min (IL-8) 166 or may take up to three weeks (chemotherapy) 167. The mechanisms responsible for progenitor cell peripheralisation are not well
known and may be different for different stimuli. Recent experiments indicate that cytoadheson molecules may play an important role.

Hematopoietic recovery from myelosuppressive chemotherapy is associated with a transient increase in circulating progenitors. Single high-dose cyclophosphamide or other cytotoxic regimens thus enhances the collection of blood progenitor cells for autologous transplantation. Mobilisation outcome after chemotherapy relates to the degree of myelosuppression. Combining chemotherapy and HGFs (G-CSF, GM-CSF, IL-3+GM-CSF) have synergistic effects, and this strategy is today widely used for mobilisation of progenitor cells in cancer patients.

A number of different HGFs (i.e. G-CSF, GM-CSF, EPO, IL-1, IL-3, IL-8, IL-11, SCF) are capable of mobilising blood progenitor cells. Some of these agents are obviously not sufficiently effective (EPO, IL-3) or too toxic (IL-1) to become clinically useful as single agents. Combinations of different cytokines, such as G-CSF and SCF, may act synergistically.

Clinical experience of HGF alone for mobilisation of PBPC is mainly confined to G-CSF and GM-CSF. Recent findings indicate that G-CSF mobilises CD34+ cells more efficiently than GM-CSF, although GM-CSF appears to mobilise a higher proportion primitive CD34+ cell subsets (CD34+CD38-). Due to its lower toxicity, G-CSF is preferred in healthy donors.

**Mobilisation with G-CSF**

Daily administration of G-CSF significantly increases the number of circulating progenitors, including cells of the granulocyte-macrophage, erythroid and megakaryocyte lineages. Thus, Dürrsen et al observed an up to 100-fold increase in colony-forming cells in cancer patient receiving G-CSF at the dose of 3 μg/kg/d or higher. Typically, progenitor cells start to increase 2-3 days after initiating G-CSF peaking another two days later. Extending G-CSF treatment beyond 6-7 days does not further improve the mobilisation. A correlation between the dose of G-CSF and increase in progenitor cells has been shown in animals, although human data are conflicting.

A minority of patients mobilises poorly in response to G-CSF (or other regimens). Previous chemotherapy and pelvic irradiation, respectively, adversely effect the outcome of stem cell mobilisation. In healthy donors, factors predicting for low yield of CD34+ cells are not defined, although some studies suggest a negative correlation with age.

Previous reports suggest that G-CSF mobilises exclusively highly committed progenitors of the granulocyte and monocyte lineages. However, several recent studies also demonstrate mobilisation of phenotypically primitive progenitor cells (CD34+CD38-) and the capacity of G-CSF mobilised progenitors to generate and sustain hematopoiesis in vitro has been reported comparable with BM cells on a cell to cell basis. G-CSF mobilised progenitor cells have the capacity to sustain syngeneic engraftment in myeloablated mice.
Collection of BPC
Hematopoietic stem cells coseparates with monocytes and lymphocytes on centrifugal gradients. The technology of large-scale mononuclear cell collection by continuous flow blood cell separation was developed during the 1970s and early 1980s. A typical leukapheresis session implies the processing of 2-3 times the normal blood volume with a flow rate of 50-80 ml/min and a collection efficiency of usually 50-70%. Peripheral veins or various forms of dual lumen venous catheters are used as vascular access. Major side-effects of the leukapheresis procedure are uncommon, although non-trivial catheter related complications occasionally occur.

Enumeration of BPC
The CD34 antigen is expressed by hematopoietic progenitor cells, endothelial cells and myeloid tumor cells. Hematopoietic cells expressing CD34 includes both primitive and committed progenitor cells. Progenitors capable of forming colonies in semisolid culture assays reside in the CD34+ population. CD34+ selected bone marrow and blood cell grafts, respectively, are capable of reconstituting hematopoiesis in patients receiving myeloablative chemotherapy.

Flow cytometric enumeration of CD34+ cells, using fluorochrome-conjugated anti-CD34 mAbs, has become a standard method for evaluating the quality of blood cell grafts and for monitoring of progenitor cell mobilisation. Several reports confirm a good correlation between numbers of CD34+ cells and CFU-GM. Although CD34-measurements have less interlaboratory variability than the CFU-GM assay, lack of standardisation still makes it difficult to compare results from different centers.

In the setting of autologous blood progenitor cell transplantation, the number of CD34+ cells infused correlates with the rate of hematopoietic (neutrophil and platelet) reconstitution. However, increasing the dose of progenitor cells above a certain "upper threshold" may not further improve the rate of hematopoietic recovery. Blood cell grafts containing less than 1-2.0x10^6 CD34+ cells/kg tend to be associated with delayed platelet recovery.

The majority of CD34+ cells are lineage committed. Primitive progenitors may be enumerated using the LTC-IC assay or other in vitro culture methods. However, these methods are too complicated for routine clinical use. Alternatively, phenotypically primitive cells may be quantified by multiparameter flow cytometry. Thus, primitive CD34+ cell subsets are defined by the lack of expression of differentiation (CD33 etc) or activation (CD38, HLA-DR) antigens, or by the coexpression of CDw90 (Thy-1).
Autologous BPCT

Early experience of autologous BPCT was mainly derived from patients not eligible for bone marrow harvesting due to previous pelvic irradiation or tumor infiltration of the marrow. Collection of steady-state blood stem cells typically required median eight leukaphereses to obtain a yield considered sufficient for transplantation. Hematopoietic reconstitution following transplantation of unmobilised BPCT was comparable to ABMT with occasional patients experiencing delayed engraftment. Mobilisation with chemotherapy and/or cytokines makes it possible to collect a larger number of blood stem cells than is contained in a bone marrow harvest, using a limited number of leukaphereses. Consequently, high-dose chemotherapy supported with mobilised PBPC is associated with more rapid lymphocyte, neutrophil and, in particular, platelet recovery compared with ABMT. A reduced need of platelet transfusions and shorter time in hospital post transplant has been shown in unrandomised studies. Thus, blood cell grafts have gradually replaced bone marrow in most categories of patients undergoing high-dose chemotherapy. Paradoxically, the original hypothesis that blood cell transplants are less likely to be contaminated by tumour cells is yet unproven.

Allogeneic BPCT

Allogeneic BPCT may have advantages over allogeneic BMT in terms of more rapid hematopoietic and/or immunological reconstitution. The possibility to obtain a larger number of progenitors may be particularly important in the settings of graft failure and donor-receptor mismatch, respectively. In some donors, the avoidance of bone marrow harvest/general anaesthesia might be preferable. Despite these potential advantages, allogeneic PBCT did not appear on the clinical arena until very recently. Reasons for this include reluctance to mobilise healthy donors with G-CSF, uncertainty about long-term engraftment capacity of blood stem cells and the supposed increased risk of GVHD. G-CSF mobilised blood cell grafts contain about 1 log more T-cells than an average bone marrow graft. Theoretically, the high number of T-cells (and NK-cells) may be advantageous from the point view of GVL. Whilst murine studies demonstrate a correlation between the number of infused T-cells and development of GVHD, this has not been uniformly shown in humans. Thus, it has been hypothesised that the risk of GVHD might not increase above a certain threshold level of T-cells.

The possible effects of G-CSF on T-cells are of interest in this context. Thus, in a recent study in mice, it was shown that treating donors with G-CSF modulates donor T-cells toward production of type-2 cytokines, which is associated with a reduced severity of acute GVHD. Survival was improved in recipients of G-CSF treated donors.

The number of hematopoietic progenitor cells required for rapid and sustained allogeneic engraftment has not yet been established, present recommendations...
of ≥4-5 CD34+ cells/kg being based on extrapolations from the autotransplant setting. In healthy donors, such a number of progenitors may be collected by 1-3 leukapheresis after mobilisation with G-CSF 5-16 μg/kg/d for 4-6 days. G-CSF has been generally well tolerated, although "bone pain" seems to be more frequent in normal donors than in cancer patients receiving G-CSF. The optimal dosage of G-CSF for mobilisation of BPC in healthy donors has not yet been defined.

Clinical experience of alloBPCT is rapidly growing. Recently published small studies suggest that sibling donor transplantation of blood progenitor cells restores hematopoiesis at least as rapid as bone marrow allografts without increasing the risk of acute GVHD. Sustained allogeneic engraftment has been documented up to two years post transplant. The impact of BPCT on the incidence/severity of chronic GVHD and disease-free survival, respectively, is yet not known. This, as well as the value of T-cell depletion by CD34-enrichment, is being evaluated in ongoing Phase III trials.

The effect of rGM-CSF on neutrophil and eosinophil regeneration after ABMT as monitored by circulating levels of granule proteins. (paper I)

Rational
Blood levels of neutrophil granule proteins reflect the neutropoietic activity of bone marrow. We have previously shown that blood measurements of MPO and LF may be used to monitor the activity of regenerating neutropoiesis after ABMT.

The major aim of this study was to investigate the effects of rGM-CSF on the granulopoiesis after ABMT.

Materials and methods
Twenty-two consecutive adult patients with non-myeloid malignancies were randomised to receive either rGM-CSF 10 μg/kg or placebo from day +1 after ABMT until ANC>1x10^9/l for four consecutive days. P-MPO, S-MPO, P-LF, S-LF and S-ECP were determined daily during a period of 3-4 weeks by means of sensitive radioimmunoassays.

Results and discussion
Following neutrophil engraftment (ANC>0.1x10^9/l) at median day 11 (GM) and 12 (placebo), respectively, ANC increased more rapidly in the GM-CSF group. P-MPO and S-MPO did not differ between the two groups during the early post engraftment period (d+11-19), suggesting that GM-CSF had no effect on neutropoietic proliferation during this period. These findings suggest that GM-CSF after ABMT increases the blood number of neutrophils by other mechanisms such as prolonging the half-life in circulation and early release from bone marrow.
In the GM-CSF group, S-ECP increased during the second and third week post ABMT, which corresponded to an increase in eosinophil counts. This indicates that GM-CSF stimulates eosinophil recovery without causing an activation of the eosinophils.

**Conclusions.**
GM-CSF increases ANC after ABMT through other mechanisms than stimulation of neutropoietic proliferation. GM-CSF stimulates eosinophil recovery after ABMT.

**The effect of granulocyte colony-stimulating factor (G-CSF) on the degranulation of secondary granule proteins from human neutrophils in vivo may be indirect.**

*(paper II)*

**Rationale**
Single injections of G-CSF result in neutrophil activation and degranulation. The effects of repeated administration of G-CSF on neutrophil degranulation are less well known. LF and HNL are proteins contained in different subpopulations of the secondary granules of neutrophil granulocytes.
The aim of the present study was to investigate the effects of G-CSF on neutrophil secondary degranulation *in vivo* and *in vitro*. We were also interested in the possible involvement of other cytokines in the activation of neutrophils during administration of G-CSF.

**Material and methods.**
Healthy male volunteers (aged 21–35) received G-CSF (lenograstim) 7.5-10 μg/kg s.c. once daily for six days (day 1–6). Blood samples were obtained at day 1–8, 12 and at follow up 3±1 weeks after the last injection. HNL and LF were determined in serum and plasma by RIA, and TNF-α (free TNF-α+TNF-α bound to soluble receptors in samples) in plasma using a commercial ELISA. Granulocytes were separated from heparinised blood by density gradient centrifugation over 67% Percoll, and assayed for intracellular content of HNL and LF, respectively. Separated granulocytes were stimulated with FMLP and PMA, and the supernatants assayed for granule proteins (HNL, LF, MPO).

**Results and discussion.**
Following G-CSF 7.5 μg/kg sc (n=8), blood HNL, LF and neutrophils increased with a similar kinetics, i.e. an initial rapid rise was followed by a plateau day 2–7 and a subsequent return to baseline.
At day 5, separated neutrophils showed an enhanced release of HNL and LF, respectively, in response to f-MLP compared to neutrophils obtained at baseline (day 1). By contrast, f-MLP induced release of HNL and LF, respectively, was only marginally enhanced by preincubation of normal neutrophils with G-CSF *in vitro*. Taken together, these findings indicate the effect of G-CSF on secondary degranulation is mainly indirect.

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Following G-CSF 10 μg/kg (n=2), P-TNF-α increased about 25-fold. TNF-α started to increase at day 2 and peaked at day 6. Intriguingly, the elevated P-TNF-α levels were temporarily correlated to other signs of neutrophil activation. We therefore speculate that TNF-α may be partly responsible for the activation of neutrophils during administration of G-CSF.

Conclusions
G-CSF enhances release of secondary granule proteins from neutrophil granulocytes in vivo. The effect of G-CSF on secondary degranulation is mainly indirect. G-CSF induces an increase in P-TNF-α.

Effects of in vivo administration of G-CSF on neutrophil functions in healthy volunteers
(paper III)

Rationale
G-CSF has multiple effects on mature neutrophil function in vitro and in vivo. Previous studies of in vivo effects of G-CSF on neutrophil function have given conflicting results, particularly with respect to chemotaxis. G-CSF is increasingly used in healthy donors to enhance the collection of granulocytes for transfusion.

The major aim of this study was to further characterise the effects of G-CSF, administered as repeated s.c. injections to healthy volunteers, on neutrophil functions.

Materials and methods
G-CSF was administered s.c. once daily for six days (day 1-6) to healthy male volunteers. Neutrophil migration (modified Boyden chamber), chemiluminescence (CL), adherence to nylon fibers and phagocytosis of IgG- and IgG-C3-coated particles were investigated before, day 2, day 5 and three weeks after G-CSF 7.5-10 μg/kg/d (n=12). Neutrophil surface expression of adhesion molecules and Fcγ-receptors, respectively, was measured by flow cytometry before, day 5, day 8 and three weeks after G-CSF 3-5 μg/kg/d (n=12). Results obtained after G-CSF were compared to baseline using Wilcoxon’s signed rank test.

Results and discussion
G-CSF decreased neutrophil chemotaxis towards zymosan activated serum, f-MLP and IL-8, respectively. Chemotaxis was reduced both at day 2 and 5. The negative effective on chemotaxis could only partly be explained by a reduced chemokinesis
G-CSF increased neutrophil adherence and phagocytosis of both IgG-coated and IgG-C3-coated particles.
G-CSF (3-5 μg/kg) enhanced the neutrophil surface expression of adhesion molecules (CD11b/CD18, CD35) and of Fcγ-receptors (CD32, CD64), respectively.
G-CSF had no effect on lucigenin enhanced CL, irrespective of whether the neutrophils had been activated by PMA or serum-opsonised zymosan. Contrary to previous reports, this suggests that G-CSF in vivo has no effect on the O2- producing enzyme complexes of the respiratory burst.

Conclusions,
G-CSF in vivo impairs neutrophil chemotaxis but enhances neutrophil adherence and phagocytosis. G-CSF in vivo does not stimulate the respiratory burst.

G-CSF reduces neutropenia but not infectious morbidity during induction therapy for acute lymphoblastic leukemia
(paper IV)

Rational
Remission induction chemotherapy for adult ALL is associated with a high incidence of infections during the neutropenic phase. The aim of this study was to assess the value of G-CSF (lenograstim) in reducing neutropenia (duration, severity) and infectious morbidity (incidence of severe infection, duration of fever) in adult patients with ALL receiving remission induction chemotherapy.

Materials and methods.
Consecutive patients with newly diagnosed ALL were randomised to receive (n=41) or not receive (n=39) G-CSF (lenograstim) during 28 days of remission induction chemotherapy. G-CSF 5 μg/kg was administered as sc injections once daily at days 3-14 and 17-28. All patients were hospitalised and received prophylactic antibiotics (norfloxacin) during this period. Blood counts, fever and clinical symptoms were assessed daily.

Results and discussion.
G-CSF was well tolerated and did not appear to adversely affect disease outcome. Compared to the untreated control group, G-CSF reduced the duration of severe neutropenia (ANC<0,5x10^9/l) from 20 to 14 days (p=0.0135). However, G-CSF had no impact on the incidence of severe infections (septicaemia, pneumonia, abscess), days with fever nor days with i.v. antibiotics.

The incidence of severe infections in this study was (in both arms) only about 20% compared to 45% in a previous trial with the same chemotherapy. Improved supportive care including use of prophylactic antibiotics might explain this difference.

The "discrepancy" between the effect of G-CSF on neutropenia and the lack of impact on infectious morbidity is consistent with the findings of other studies of G-CSF after chemotherapy. We cannot exclude that subgroups of ALL patients might benefit from the addition of G-CSF. However, based on the
results of this study we cannot recommend the routinely use of G-CSF during remission induction chemotherapy for ALL.

Conclusions
G-CSF shortens the duration of neutropenia but does not reduce infectious morbidity in patients with ALL undergoing remission induction chemotherapy.

Dose Dependent Mobilisation of Haematopoietic Progenitor Cells in Healthy Volunteers Receiving Glycosylated rHuG-CSF (paperV)

Rational
The optimal dosage of G-CSF for blood progenitor cell mobilisation in healthy donors has not been defined. Previous studies of a possible dose-response relationship have given conflicting results. It is not clear to which extent G-CSF mobilises primitive progenitor cells.
The primary objective of this trial was to study whether increasing the dose of lenograstim (glycosylated G-CSF) improves the mobilisation of progenitor cells in healthy volunteers. Major secondary objectives were to investigate the G-CSF induced mobilisation of primitive CD34+ cell subsets, and the safety and tolerability of lenograstim when administered to healthy subjects.

Materials and methods
Twenty-four healthy male volunteers, aged 20-34 years, were included. Four groups of six volunteers received lenograstim once daily for six days (days 1-6) at a dose of 3, 5, 7.5 and 10 µg/kg/d s.c., respectively. All subjects underwent a 10-L leukapheresis on day 5, 6 or 7. CD34+ cells were enumerated by flow cytometry using directly conjugated mab (HPCA-2). CD34+ cells in bone marrow (BM) aspirates obtained prior to G-CSF and in leukapheresis products (LP), respectively, were further immunophenotyped.

Results and discussion
Lenograstim was overall well tolerated. The majority of volunteers reported bone pain, which tended to be more intense in the group receiving 10 µg/kg.
The intensity of bone pain and the extreme leukocytosis (WBC> 60x10^9/l) in some volunteers receiving 10 µg/kg prevented further escalation of lenograstim.
Maximal mobilisation of progenitor cells occurred on day 5 or 6. Lenograstim 10 µg/kg produced significantly higher peak levels of CD34+ cells, BFU-E and CFU-GM, respectively, compared with each of the other doses (p<0.05, ANOVA). A good correlation was observed between the number of CD34+ cells in blood and apheresis product, respectively, (r=0.95, p=0.0001).
A comparison of steady state BM CD34+ cells and LP CD34+ cells in paired samples for each individual, showed a higher proportion of primitive CD34+ cell subsets (CDw90+, HLA-DR-, CD45RA-, CD33-) among LP CD34+ cells.
Taken together these results suggest that lenograstim 10 µg/kg/d is an optimal dose for mobilisation of progenitor cells in healthy donors. Corroborating our findings, a recent study showed that filgrastim 10 µg/kg in healthy volunteers was tolerable and mobilised progenitor cells more efficiently than 3-5 µg/kg. None of these two studies have explored dose levels above 10 µg/kg.

Conclusions
Lenograstim 10 µg/kg/d to healthy volunteers is well tolerated and mobilises progenitor cells more efficiently than 3, 5 and 7.5 µg/kg/d, respectively. G-CSF favors mobilisation of primitive CD34+ cell subsets. Lenograstim 10 µg/kg/d for 5-6 days should provide an efficient mobilisation of progenitor cells in most healthy donors.

Glycosylated G-CSF is more potent than non-glycosylated G-CSF in mobilisation of peripheral blood progenitor cells in healthy volunteers

(paper VI)

Rational
In vitro bone marrow colony assay studies indicate that lenograstim (glycosylated G-CSF) is more potent than filgrastim (non-glycosylated G-CSF). However, such a difference has not yet been shown in vivo. The primary objective of this trial was to compare the efficacy of equivalent doses (µg) of lenograstim and filgrastim G-CSF in mobilising CD34+ cells. Major secondary objectives were to compare the kinetics of the increase in CD34+ cells in blood, and to assess the safety and tolerability of the two drugs given to healthy subjects.

Materials and methods
Thirty-two healthy male volunteers, median age 27 years (19-44), were randomised to receive either lenograstim 10 µg/kg followed by filgrastim 10 µg/kg or vice versa with a wash-out period of minimum four weeks. Both drugs were administered as s.c. injections once daily for five days (day 1-5). CD34+ cells were enumerated at days 1 and 4-8. All flow cytometric analyses were performed blindly by one and the same investigator.

Results and discussion
There was no evidence of a carry-over effect for any of the predefined efficacy variables. CD34+ cells were mobilised with a similar kinetics, peaking at median day 6 (5-6) for both drugs. A significant difference in favor of lenograstim was shown for peak number of CD34+ cells/µl blood (104±38 vs 82±35, mean±1SD, p<0.0001, paired t-test, n=30) and number of CFU-GM/µl blood at day 6 (14.6±8.4 vs 10.2±4.6, p<0.0001), respectively. There was no difference in day 6 number of CD3+ cells. Both drugs were generally well tolerated and did not differ with respect to number of adverse events.
The results show that there is a difference in in vivo potency between lenograstim and filgrastim. It seems reasonable to assume that the greater potency of lenograstim is related to its being glycosylated, although the physiological significance of the carbohydrate chain of G-CSF molecule is unknown. As for clinical implications, the finding may be relevant for the dosing of G-CSF for mobilisation of progenitor cells in healthy donors.

Conclusion.
Lenograstim 10 µg/kg/day mobilises blood progenitor cells more efficiently than filgrastim 10 µg/kg/day in healthy volunteers, indicating a difference in in vivo potency between the two G-CSFs.

**GENERAL CONCLUSIONS**

**GM-CSF after ABMT**
- increases neutrophil counts through other mechanisms than stimulation of neutropoietic proliferation
- stimulates eosinophil regeneration

**G-CSF in vivo**
- enhances the release of secondary granule proteins from neutrophil granulocytes
- increases the plasma level of TNF-α
- impairs neutrophil chemotaxis
- enhances neutrophil adherence and phagocytic capacity
- has no effect on the respiratory burst

**G-CSF during standard induction chemotherapy for ALL**
- reduces the period of neutropenia
- no difference in infectious morbidity could be shown

**G-CSF 3-10 µg/kg/d to healthy volunteers**
- favors mobilisation of primitive CD34+ cell subsets

**Glycosylated G-CSF (lenograstim) 10 µg/kg/d to healthy volunteers**
- is well tolerated
- mobilises progenitor cells more efficiently than 3, 5 and 7.5 µg/kg/d
- mobilises progenitor cells more efficiently compared to an identical (µg) dose of non-glykosylated G-CSF (filgrastim)
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A complete list of references may be obtained from the author.