Discovery of G-CSF and Early Clinical Studies

Karl Welte

1 Introduction

In the 1960s, two groups simultaneously developed methods for growing colonies of granulocytes and monocytes from mouse bone marrow or spleen cells in semi-solid agar (for review, see [1]). The colony growth was dependent on the presence of unknown factors, which were given the operational name colony-stimulating factors (CSF). Efforts to biologically identify and biochemically purify these CSF kept many laboratories busy until the middle of the 1980s and revealed that there is no single CSF, but rather four quite biochemically different CSF with different colony-stimulating activities. The four CSF were given names dependent on the type of colonies: GM-CSF stimulated granulocyte and macrophage colonies; M-CSF, macrophage colonies; G-CSF, granulocyte colonies; and multi-CSF (interleukin [IL-3]), a broad range of hematopoietic cell colonies [1].

2 Purification and Biochemical Characteristics of G-CSF

Murine G-CSF was purified by Nick Nicola and colleagues in Melbourne, Australia, in 1983 [2], and human G-CSF was purified independently by a group, which included Erich Platzer and myself, in New York in 1983/1984 [3] (Fig. 1). The murine G-CSF was purified from mouse lung-conditioned medium, and the human G-CSF from the human bladder carcinoma cell line 5637. Intriguingly, not knowing the results of the work of Nicola et al. when we started to purify our CSF in 1983, our goal was initially to purify human IL-3, as we had been successful in

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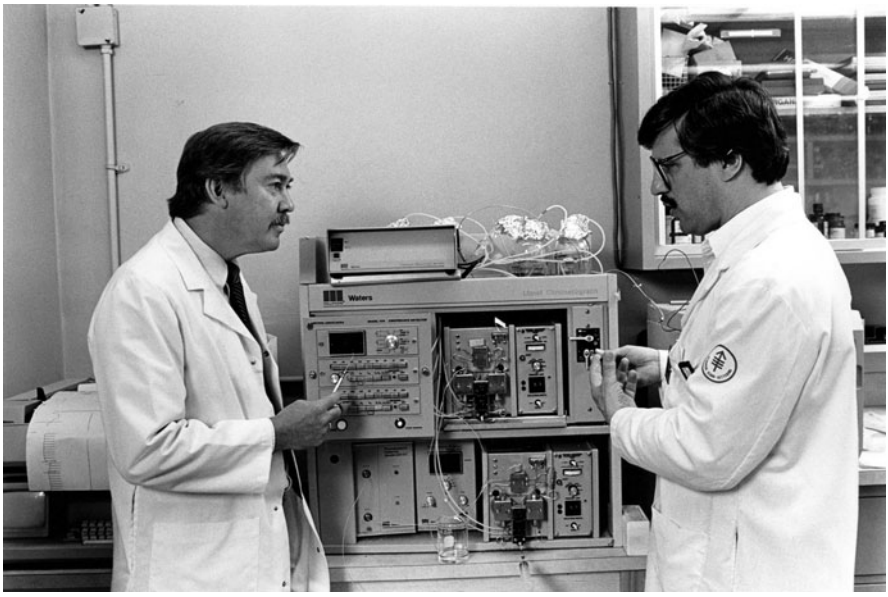

Fig. 1 Karl Welte (right) and Malcolm A.S. Moore at the HPLC equipment in the Memorial Sloan-Kettering Cancer Center, New York, NY, USA

purifying IL-2 [4] and Ihle and colleagues had recently successfully purified murine IL-3 [5]. These findings were one of the reasons why we called our CSF the first pluripotent hematopoietic CSF [3]. Starting with 40 L 5637-conditioned medium, we succeeded in purifying G-CSF to homogeneity by ion-exchange chromatography, gel filtration, and reversed-phase high-pressure liquid chromatography (HPLC), and produced 5 μg of pure G-CSF as judged by silver staining in polyacrylamide-gel electrophoresis. G-CSF is *O*-glycosylated and has a molecular weight of 19,600 Da. Because the amino acid sequence of G-CSF was not known, we asked Por Li and Lawrence Souza at Amgen, Thousand Oaks, CA, to help us to get the initial *N*-terminal amino acid sequence. Deduced from the amino acid sequence, molecular cloning of the cDNA for G-CSF and the first expression in *Escherichia coli* were achieved in 1986 by Souza and Boone at Amgen in cooperation with our laboratory [6]. The recombinant G-CSF (rG-CSF) was capable of supporting myeloid proliferation and differentiation in granulocyte-macrophage progenitor cell (CFU-GM) assays. We were also able to identify G-CSF receptors (G-CSFR) on myeloid leukemia cells by binding studies with ^{125}I-labeled G-CSF [6]. Later, in 1986, a Japanese group purified G-CSF from the cell line CHU-2 [7], and Nagata et al. [8] cloned the cDNA encoding G-CSF from the same cell line by using probes derived the partial amino acid sequence of the purified G-CSF from CHU-2 cells. However, they described a protein with 177 amino acids, which might be a splice variant of the 174-amino acid G-CSF we obtained from the 5637 cell line. In 1990, the specific G-CSFR was characterized and cloned [9] and was shown

to be a homodimer. It is interesting to note that a few hundred receptors per cell can induce optimal responses to G-CSF. The binding of G-CSF to its receptor activates many signaling pathways such as phosphorylation of JAK2, STAT3, and STAT5, and the transcription factors LEF-1 and C/EBPα [10]. An interesting study demonstrated that G-CSF leads to upregulation of the expression of nicotinamide phosphoribosyltransferase (Nampt), which induces an increase in NAD^+ and sirtuins [11].

3 Biology of G-CSF

A range of actions have been documented for G-CSF. In a CFU-GM assay, G-CSF is capable of inducing growth of mainly neutrophiilic granulocyte colonies. It also acts on the function of mature neutrophils, such as enhancement of chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) binding on mature neutrophils [12]. Granulocyte colony formation can be stimulated with GM-CSF, M-CSF, IL-3, and stem cell factor (SCF). An interesting study compared native G-CSF and rG-CSF and showed that they had identical biologic activities [13]. The study showed that the removal of monocytes and T-lymphocytes from the bone marrow abrogated the growth of erythroid progenitor cells (BFU-E) and granulocyte–erythrocyte–monocyte–megakaryocyte progenitor cells (CFU-GEMM) when native G-CSF was used as a stimulator. The initial term "pluripotent CSF" (PPO) was created because native purified G-CSF stimulated also BFU-E and CFU-GEMM in unseparated bone marrow due to stimulation of monocytes and T-lymphocytes to release GM-CSF [13]. The solution of the specificity of the single CSF came from gene deletion studies in mice. G-CSF knock-out studies revealed that it is clearly responsible for proliferation and differentiation of granulocyte progenitor and precursor cells [14]. Mice lacking endogenous G-CSF have chronic neutropenia and impaired neutrophil mobilization, indicating that G-CSF is indispensable for maintaining the normal balance of neutrophil production during steady-state myelopoiesis.

4 Nonclinical In Vivo Studies with G-CSF

Soon after the availability of rG-CSF, its potential clinical use was investigated. We investigated the effects of rG-CSF in nonhuman primates (cynomolgus monkeys) [15]. After subcutaneous administration, the white blood cell counts increased in a rG-CSF dose-dependent manner 24 h after initiation of treatment, and with daily treatment reached a plateau by day 6. The increase in the white blood cell count was mainly due to the increase in neutrophil counts. For example, at 10 μg/kg/day, the absolute neutrophil count (ANC) increased to approximately 50×10^9/L and at a dose of 100 μg/kg/day, the count increased to approximately 100×10^9/L.

The neutrophil counts could be maintained for the duration of a 4-week treatment period and returned to normal values 3 days after treatment ended. The absolute numbers of lymphocytes increased approximately twofold, but the number of monocytes, eosinophils, reticulocytes, and platelets did not change significantly. In the spleen, foci of extramedullary hematopoiesis were observed. No other organs demonstrated evidence of hematopoietic activities. The neutrophils functioned normally in standard tests for chemotaxis and there was evidence of enhanced ability to kill phagocytized bacteria [15].

We further investigated whether or not rG-CSF might be of benefit in chemotherapy-induced neutropenia and after bone marrow transplantation. rG-CSF was able to shorten the period of chemotherapy-induced neutropenia after high-dose cyclophosphamide and busulfan, or total body irradiation followed by autologous bone marrow transplantation [15, 16].

These results demonstrated that G-CSF is a potent granulopoietic growth and differentiation factor in vivo and opened the avenue of the use of rHuG-CSF in the treatment of patients not only with chemotherapy-induced neutropenias but also with other clinical situations associated with chronic neutropenias.

5 Clinical Uses of rHuG-CSF

5.1 Phase 1 and Phase 2 Studies

The first clinical use of recombinant human G-CSF (rHuG-CSF) was performed at the Memorial Sloan-Kettering Cancer Center, New York, in patients with transitional cell carcinoma of the urothelium in 1987 [17]. The study was designed as an open-label phase 1/2 study of both the safety and efficacy of five dosages of rHuG-CSF, with each dosage evaluated in three to five patients receiving the M-VAC chemotherapy (methotrexate, vinblastine, doxorubicin, and cisplatin). The dosages were 1, 3, 10, 30, and 60 μg/kg/day subcutaneously, given before (day 12 to day 7) or during the first cycle on day 4 through day 11 of treatment with M-VAC, or both. Treatment with rHuG-CSF before chemotherapy produced a dose-dependent increase in neutrophil counts, and the use of rHuG-CSF after M-VAC chemotherapy significantly reduced the number of days per patient on which the ANC was $\leq 1.0 \times 10^9$/L, reduced the number of days on which antibiotics were used to treat febrile neutropenia, and significantly increased the percentage of patients qualified to receive planned chemotherapy. These findings demonstrated that rHuG-CSF can reduce both the hematopoietic and oral toxicity of chemotherapy [17].

A second phase 1/2 study used rHuG-CSF in 12 patients who were receiving intensive chemotherapy for small-cell lung cancer [18]. Patients were treated by continuous infusion of rHuG-CSF at 1, 5, 10, 20, or 40 μg/kg/day for 5 days before chemotherapy and 14 days after adriamycin, ifosfamide, and etoposide

chemotherapy on alternative cycles. As in the first study [17], use of rHuG-CSF reduced the period of neutropenia considerably and no infectious episodes were observed in the cycles when patients received rHuG-CSF [18].

5.2 Phase 3 Studies

Two randomized multicenter studies with rHuG-CSF were performed in patients with small-cell lung cancer in the USA [19] and in Europe [20]. These studies were designed to test whether or not rHuG-CSF could decrease the incidence of infections as manifested by febrile neutropenia, and whether or not the use of rHuG-CSF would lead to a reduction in the incidence of intravenous antibiotic use, hospitalization, and culture-confirmed infections. Both studies used chemotherapy with cyclophosphamide, doxorubicin, and etoposide. The reduction in febrile neutropenia episodes was similar in both studies, and no statistically significant differences were detected in tumor response rates or overall survival.

5.3 Use of rHuG-CSF in Hematologic Malignancies

The use of rHuG-CSF in patients with acute myeloid leukemia (AML) remained controversial because of the in vitro observation that leukemic cells express G-CSFR [6] and that G-CSF could stimulate leukemic cell growth [21]. A prospective, randomized study was done to determine the efficacy and safety of rHuG-CSF after standard intensive chemotherapy in 108 patients with relapsed or refractory acute leukemia [22]. Treatment with rHuG-CSF was shown to accelerate the recovery of neutrophils and reduce the incidence of infections. No difference was observed in remission rates between the two treatment groups. The researchers further explored whether or not the administration of rHuG-CSF before and during chemotherapy would result in enhanced killing of leukemic cells. No difference was seen between the groups in event-free survival or in disease-free survival in patients who did achieve a complete remission. Another group investigated the use of rHuG-CSF before and during chemotherapy in AML patients and did not see any effect on complete remission [23]. The ability of rHuG-CSF to induce remission by stimulating residual normal donor cells in patients after allogeneic stem cell transplantation was studied [24]. The investigators reported that rHuG-CSF might be effective in selected patients with early relapse after allogeneic bone marrow transplantation.

We conducted a randomized study with rHuG-CSF in children with high-risk acute lymphoblastic leukemia (ALL) treated according to the ALL-BFM chemotherapy protocol [25]. Children were randomly assigned to receive nine alternating cycles of chemotherapy alone or followed by rHuG-CSF at 5 μg/kg/day. In both groups, the

planned interval between chemotherapy courses was 21 days. Of the 34 patients analyzed, the incidence of febrile neutropenia, the number of culture-confirmed infections, and the total duration of intravenous antibiotic treatment were reduced significantly; however, with a median follow-up of 15 years, no difference was observed with regard to the estimated event-free survival [25].

5.4 Use of rHuG-CSF in Stem Cell Transplantation

Myeloablative chemotherapy requires cellular reconstitution, and rHuG-CSF alone is not sufficient to produce recovery. Because prolonged neutropenia occurs after stem cell transplantation, this setting was one of the first in which rHuG-CSF was studied. An early study administered rHuG-CSF by 30-min bolus infusion at a dose of 60 μg/kg/day beginning 24 h after autologous marrow infusion in 18 patients with Hodgkin's disease [26]. Recovery to neutrophil counts of 1×10^9/L occurred 14 days earlier in rHuG-CSF-treated patients compared with that in control patients [26]. Other researchers administered rHuG-CSF as a continuous subcutaneous infusion (20 μg/kg/day) after autologous bone marrow transplantation in patients with relapsed Hodgkin's disease, non-Hodgkin's lymphoma, ALL, and germ cell tumors [27]. The median time to an ANC $>0.5 \times 10^9$/L was 11 days compared to 20 days in the historical control patients.

5.5 Mobilization of Peripheral Blood Progenitor Cells

During the first clinical trials of rHuG-CSF in patients with cancer, an unexpected observation was made: The patients developed a 100-fold increase in the frequency of colony-forming progenitor cells in the peripheral blood. Dührsen et al. [28] were among the first to report the increases in various clonogenic hematopoietic progenitor cells on day 5 of rHuG-CSF treatment in patients with cancer. These results have been confirmed in subsequent studies [29, 30].

Sheridan et al. examined the ability of rHuG-CSF-mobilized peripheral blood progenitor cells (PBPC) to reconstitute hematopoiesis in 17 patients with poor-prognosis nonmyeloid malignancies. Of the 17 patients, 14 received high-dose chemotherapy, and the cryopreserved apheresis product was infused on day 0, followed by rHuG-CSF at 24 μg/kg/day starting on day 1. Platelet recovery was significantly faster in the rHuG-CSF-mobilized PBPC-treated patients than in historical controls [29]. Bensinger et al. studied the feasibility of using rHuG-CSF to mobilize granulocytes in normal donors and showed that rHuG-CSF was safe to administer in healthy adults [31]. In many subsequent studies, rHu G-CSF-mobilized PBPC were used instead of bone marrow stem cells in allogeneic stem cell transplantation and led to a paradigm change in stem cell transplantation. rHuG-CSF-mobilized PBPC from healthy donors have become the dominant cell

populations used in transplantations to patients with leukemia and cancer. More information about the use of rHuG-CSF in the setting of PBPC transplantation is provided in the chapter "Use of rHuG-CSF in Peripheral Blood Progenitor Cell Transplantation" by Beligaswatte et al.

5.6 *Use of rHuG-CSF in Patients with Severe Chronic Neutropenia*

One of the first clinical studies with rHuG-CSF was performed in the late 1980 in patients with congenital neutropenia at the Memorial Sloan-Kettering Cancer Center, New York, by Bonilla et al. [32]. Children born with severe congenital neutropenia are diagnosed with neutropenia at birth or shortly thereafter, which is generally accompanied by frequent and often life-threatening infections. Initially, five patients were treated with rHuG-CSF in an attempt to reduce the morbidity and mortality associated with this disorder. Patients were treated with rHuG-CSF dosages between 3 and 60 μg/kg/day administered as a continuous subcutaneous infusion. In all five patients, an increase in the number of neutrophils was reached 1 week to 9 days after the initiation of the effective dose of rHuG-CSF. All patients had sustained neutrophil counts [32]. In subsequent phase 1/2 studies with rHuG-CSF in the setting of congenital neutropenia, hundreds of patients were enrolled worldwide and a Severe Chronic Neutropenia International Registry (SCNIR) was established [33–36]. More than 90% of the patients with severe chronic neutropenia (SCN) responded to the treatment with rHuG-CSF with an increase in ANC $>1.0 \times 10^9$/L, and in most patients, the bacterial infections and the requirement for intravenous antibiotic use were significantly reduced. The subcutaneous dose of rHuG-CSF necessary to reach and maintain this ANC varied from patient to patient, and ranged between 1 and 80 μg/kg/day. The initial patients have been treated for >20 years daily without exhaustion of myelopoiesis and without generation of anti-G-CSF antibodies. Both the prognosis and the quality of life of patients with congenital neutropenia improved dramatically after the introduction of rHuG-CSF therapy in 1987. Since the establishment of the SCNIR in 1994, data on >1,000 patients have been collected worldwide to monitor the clinical course, treatment, and disease outcomes. Current knowledge of the underlying pathomechanisms suggests that congenital neutropenia is a heterogeneous multigene disorder of myelopoiesis. Genetic analyses revealed mutations in the genes for ELANE, HAX1, G6PC3, and many others. It is now well accepted that congenital neutropenia is a preleukemic syndrome and an approximately 20% of these patient develop leukemia. Independent of the genetic subtype, conversion to leukemia in congenital neutropenia is associated with acquired genetic somatic aberrations, such as G-CSF receptor mutations, monosomy 7, and *ras*-mutations [37]. More information concerning the development and use of rHuG-SCF in the setting of SCN is provided in the chapter "rHuG-CSF for the Treatment of Severe Chronic Neutropenia" by Dale and Boulyard.

6 Summary and Conclusion

The identification, purification, and molecular cloning of rHuG-CSF in the 1980s; the nonclinical studies in the mid-1980s; and the subsequent development of rHuG-CSF as a therapeutic agent in the late 1980s and 1990s have had a major influence on the treatment of many diseases. rHuG-CSF has been the topic of many clinical researches and publications in the last 20 years. rHuG-CSF has specific and selective actions due to the restricted expression of G-CSFR on myelopoiesis. It stimulates proliferation, differentiation, and activation of cells of the neutrophil lineage. In the clinical setting, rHuG-CSF is of benefit to patients receiving chemotherapy or myeloablative treatment. It has been shown to reduce morbidity in many patient populations. Stem cell transplantation using rHuG-CSF-mobilized PBPC revolutionized stem cell transplantation, making it simpler, more efficient, and more widely applicable in the clinic. However, clinical research is still necessary to improve the use of rHuG-CSF in patients with cancer and other diseases.

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