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The CSFs and Cancer

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Abstract

The four colony stimulating factors (CSFs) are glycoproteins regulating the generation and some functions of infection-protective granulocytes and macrophages. Recombinant G-CSF and GM-CSF have now been used to elevate dangerously low white blood cell levels in many millions of cancer patients following chemotherapy. These CSFs also release haematopoietic stem cells to the peripheral blood (PBSC) and these cells have now largely replaced bone marrow as more effective populations for transplantation to cancer patients with treatment-induced bone marrow damage.

When I began my career in leukaemia research in 1954, most workers in the field were searching for human leukaemia viruses. My interest in the quite different field of blood cell regulators had been aroused by work with tumours of endocrine target tissues, such as the thyroid or breast¹. In elegant studies, Furth had shown that if mice were subjected to a sustained imbalance in hormones favouring cell proliferation, tumour development occurred in a stepwise fashion in the target tissues^{2,3}. When thinking about how leukaemia might initiate, I was intrigued by the ideas of Furth in his 1954 essay: “On the basis of events with other regulated cells it can be postulated that a permanent disturbance of the homeostatic balance might result in leukaemias in which the proliferating cells are essentially unaltered, and which could be controlled at their inception by restoration of the deranged equilibrium of the regulatory forces.”³. In the context of leukaemia, although commonsense said that regulators must exist to control white blood cells, unfortunately nothing was known about the possible nature of these regulators.

Discovery, purification and cloning of the CSFs

Prior to the 1960s, many investigators had performed experiments in intact animals to discover possible regulators of white blood cell homeostasis, but nothing of substance had been observed. The situation changed dramatically in 1965–66 when two groups simultaneously developed methods for growing colonies of white blood cells from mouse bone marrow or spleen cells in semi-solid agar and later in methylcellulose cultures^{4,5}. The colonies, as initially grown, contained maturing neutrophilic granulocytes (hereafter simply called granulocytes or neutrophils) and/or macrophages. The remarkable features of these colonies were that they were clones derived from single precursor cells (later termed progenitor cells (Box 1)) and that the formation, number and size of colonies were absolutely dependent on the amount of cells, tissue extracts, or medium conditioned by various tissues that were added to the cultures^{4,6}. The culture system clearly was dependent on the presence of an unknown active factor(s) (given the operational term, colony-stimulating factor, CSF)⁷ that was needed to stimulate cell division. Subsequent efforts succeeded in growing comparable colonies from human marrow cells, using underlayers of white blood cells as ‘feeder layers’ that provided a source of the as yet unknown CSF⁸.

Initial studies indicated that CSF was probably not a virus that had transformed marrow cells (at that time only transformed cells were believed capable of proliferation in agar medium), was not some trivial nutritional material, and was probably a protein. Efforts to purify CSF

occupied many laboratories during 1968–1985. Initially human urine was used as source material⁹, then mouse organ or cell line conditioned medium and eventually comparable media from human cells or human tumour cell conditioned media were used. The task proved to be formidable. It slowly became inescapable that there was not a single CSF but, in fact, there were four quite different glycosylated CSF proteins each with differing colony stimulating activity. The task of separating and purifying these four CSFs was rendered much more difficult by variable glycosylation of the CSFs and the minute amounts of CSF in tissues. The four CSFs were given working names that indicated the most numerous type of colony stimulated – GM-CSF (also known as CSF2), stimulating granulocyte and macrophage colony formation; M-CSF (also termed CSF1), stimulating macrophage colony formation; G-CSF (also known as CSF3), stimulating granulocyte colony formation; and multi-CSF (now more commonly termed interleukin 3, IL3) stimulating a broad range of haematopoietic cell colony types. In two instances, purification of more than half a million-fold was required to achieve pure CSF. It required the introduction and application of high-performance liquid chromatography to lead to eventual success in these purification attempts. Purification of murine GM-CSF¹⁰ and M-CSF¹¹ was reported in 1977, IL3 in 1982¹² and G-CSF in 1983¹³. Purification of human CSFs corresponding to the four murine factors followed the purifications of murine CSFs, with the investigators making better use of human tumour cell lines as superior sources of CSFs^{14–18}.

The molecular biology cloning of the cDNAs for all four CSFs, both mouse and human, from libraries using either sequence-based probes or expression screening, was achieved between 1984–1986 and were some of the earliest successes of molecular biology^{16, 19–27}. This was followed by the then difficult task of achieving expression of active protein either in bacterial, yeast or mammalian cell systems, but eventually adequate expression systems were developed.

Biology of the CSFs

The CSFs are 18–70 kDa glycoproteins and unlike the comparable erythroid regulator, erythropoietin (EPO), the CSFs are active *in vivo* in both glycosylated and non-glycosylated forms. The half-lives of glycosylated CSFs are longer than non-glycosylated CSFs, but are still only a matter of 1 to 6 hrs²⁸. Unexpectedly, CSFs were found in studies between 1966 and 1984 to be the products or potential products of most tissues and cell types in the body²⁸. Normal levels of production were very low, even in the most active tissues, but CSF production was markedly inducible by microorganisms, endotoxin or foreign cells, which could increase production up to 1,000-fold within hours²⁸. The CSFs can therefore be viewed as highly labile agents that are produced rapidly and to high levels in the presence of an inducing agent. M-CSF differs from this in that it is produced in higher concentrations in a much more stable manner. The lability and the short lifespan of CSF molecules makes the CSFs able to function as a highly responsive control system regulating haematopoietic cells.

CSFs are secreted and enter the circulation in active form. The CSFs, at times, resemble hormones, except for the multiplicity of cell types able to produce CSFs, but at other times CSFs are produced and act in a paracrine fashion in local microenvironments. Specific membrane receptors exist that are unique for each CSF and are displayed in small numbers on all maturation stages of cells in the granulocyte and monocyte-macrophage lineages from committed progenitor cells to post-mitotic mature cells in the peripheral blood and tissues (Box 1). The CSFs are removed from the circulation by binding to the specific membrane receptors displayed on granulocytic and macrophage cells, then, after internalisation of the CSF-receptor complex, are degraded^{28,29}. Degradation and/or clearance of CSFs also occurs in the liver and kidney²⁸.

The CSFs proved to be notable because of their multiple actions on haematopoietic cells (Box 2). The CSFs are mandatory to stimulate the division of every appropriate lineage-committed haematopoietic progenitor cell and its progeny. Haematopoietic progenitors exhibit great heterogeneity in their responsiveness to CSF stimulation, resulting in the characteristic sigmoid dose response curves shown in Figure 1a as more and more progenitor cells are stimulated to commence proliferation^{11–13, 28} (Box 1). Individual progenitor cells also vary in their proliferative activity but, in general, as CSF concentrations are increased, cell cycle times are shortened and there is a progressive increase in the number of progeny cells in each colony. The cells in developing granulocyte-macrophage colonies exhibit progressive maturation with time so higher CSF concentrations achieve higher numbers of mature progeny cells²⁸.

When marrow cultures were more carefully analysed and purified native or recombinant CSFs became available for more general use, it was recognised between 1977 and 1987 that agar cultures of bone marrow could sustain the development not only of granulocyte and macrophage colonies, but also colonies of eosinophils, megakaryocytes, mast cells, erythroid cells, blast cells and T and B lymphocytes. It also became apparent that the prefixes used to describe the CSFs under-represented their action. GM-CSF also stimulated eosinophil colony formation and, at high concentrations, megakaryocyte colony formation³⁰; G-CSF had a minor capacity to stimulate some granulocyte-macrophage colonies³¹. M-CSF could stimulate granulocyte colony formation by some progenitor cells²⁸ and IL3 could stimulate colony formation by progenitors of blast cells, granulocytes, macrophages, megakaryocytes, eosinophils, mast cells and erythroid cells³². When used in combination with an agent like stem cell factor (SCF, also known as KIT ligand), the CSFs could co-stimulate the proliferation of the earliest haematopoietic cells (see Box 1)^{33–35}.

It became apparent in 1967 that the CSFs were necessary for the survival of the progenitor (colony-forming) cells and their progeny in culture^{36–38} and in 1990 withdrawal of CSF was shown to lead to death from apoptosis³⁹. This initially prompted some to postulate that the CSFs had only survival effects and that cells were then able to proliferate spontaneously. This improbable suggestion was discounted by the persisting dependency on CSF for proliferation of cell lines whose survival had been ensured by overexpression of BCL2⁴⁰ and by the characterisation of some of the intracellular mitotic signals including JAK-STAT and cyclin activations initiated when CSFs interact with their membrane receptors⁴¹.

More controversial have been the findings that CSFs appear able to initiate maturation events in leukaemic cell lines^{13,42}, and possibly, at times, to dictate commitment decisions in granulocyte-macrophage precursors *in vitro*^{43,44}. These actions require further elucidation because some maturation was observed in a BCL2-immortalized cell line in the absence of CSFs⁴⁵ and CSFs can stimulate cell proliferation in unusual cell types following insertion and expression of CSF receptors into such cells⁴⁶. This type of stimulation, however, does not alter the phenotype of the cells responding to the CSF.

Finally, CSFs clearly have the capacity to stimulate the functional activity of mature cells. For example, GM-CSF can act on mature neutrophils to exhibit chemotaxis, enhance oxidative metabolism, enhance antibody-dependent phagocytosis and killing of microorganisms and produce a variety of regulatory proteins. Similar actions have been documented on eosinophils and monocytes. These actions have been noted both *in vitro* and *in vivo*. A similar range of actions has been documented for G-CSF, M-CSF and IL-3 acting on mature neutrophils or monocyte-macrophages^{28, 47–51}.

It was initially puzzling how a single agent, acting at very low molar concentrations on the few hundred receptors present on responding cells could induce such diverse changes⁵².

This problem was made more complex by the recognition that only a single type of receptor existed for each CSF and that receptors for all four CSFs can co-exist on most granulocytic and macrophage cells. The problem was resolved in the early 1990s when the specific membrane receptors for each CSF were characterised and cloned. The membrane receptors in simplest form, such as for G-CSF, are homodimers⁵³ but the receptor chains can be arranged in more complex form for agents such as GM-CSF where the heterodimeric receptor is arranged as a dodecamer complex⁵⁴. All CSF receptors exhibit specific regions in their cytoplasmic domain of their signalling chains that are able to initiate the different signalling events that are required to induce a varied range of biological responses^{55–59}.

In the period following the discovery of the CSFs, other regulators of haematopoietic populations were discovered, resulting in a confusing picture of further potential redundancies or interactions in the control system. For example, granulocyte colony formation *in vitro* can be stimulated by G-CSF, GM-CSF, M-CSF, IL3, SCF, IL-6 and weakly by IL1²⁸. In particular, the CSFs appeared to have many biological actions that were potentially overlapping or redundant and it required gene knockout studies in mice in the mid 1990s to establish that each CSF did, in fact, have actions that were exclusive to that CSF.

For example, G-CSF was clearly responsible for the formation of the 75% of granulocytes under basal conditions⁶⁰. GM-CSF, by contrast, did not appear to influence mature cell numbers. Instead, it was essential for the functional activity of macrophages, particularly those in the lung. In mice, the absence of GM-CSF or its receptor leads to alveolar proteinosis – a lung disease due to failure of local macrophages to eliminate surfactant^{61,62} and the same disease state has been noted in those humans producing neutralising autoantibodies against GM-CSF⁶³. M-CSF was necessary for the formation and function of major macrophage populations and, strangely, was necessary also for tooth eruption and successful pregnancy^{64–66}. IL3 was expected to be a regulator of major importance, but mice lacking IL3 receptors showed no obvious changes in haematopoiesis^{67,68}. Later studies in mice showed that IL3 did have significant actions in allowing satisfactory mast cell and basophil responses to parasites⁶⁹ and hapten-specific delayed-type hypersensitivity responses⁷⁰.

What has emerged from knockout studies on the CSFs and other regulators is that each regulator does have some unique actions *in vivo* but, importantly, the body design often calls for synergistic actions between two or more regulators on many haematopoietic cell. For example, the dramatic effects of G-CSF *in vivo* (see below) require the synergistic action of SCF⁷¹ and strong synergy is observable on granulocyte-macrophage progenitor cells *in vitro* between GM-CSF and M-CSF or G-CSF and on less mature blast colony-forming cells between G-CSF and SCF or SCF and IL-6^{34,72}. Conversely, some combinations are inhibitory. For example, G-CSF inhibits megakaryocyte colony formation stimulated by SCF and EPO⁷³.

One picture that has emerged from the culture of mouse bone marrow cells is that lineage-committed progenitor cells can respond to single regulators but that more immature cells require two or more regulators acting in concert before proliferation occurs (Box 1). There are exceptions, and, for example, optimal proliferation of mature megakaryocyte progenitor cells requires SCF, IL-3 and EPO⁷⁴, as does the proliferation of subsets of apparently lineage-committed progenitor cells that co-fractionate after FACS separation with stem cell and CFU-S (colony-forming unit, spleen, early progeny of stem cells) populations⁷⁵.

***In vivo* actions of the CSFs in mice**

By the early 1980s the growing evidence for the existence of multiple regulators for haematopoietic tissues raised the spectre that complex interactions between these regulators might dampen or prevent any one agent from eliciting measurable responses *in vivo*.

This was not the case with the CSFs when recombinant murine CSFs became available for testing in the mid 1980s. Injection of CSFs in mice elicited responses that were qualitatively similar to the actions observed *in vitro*. Twice daily subcutaneous injections of G-CSF within four days elicited major rises in blood neutrophil levels following increased production of granulocytes in the bone marrow⁷⁶. Intraperitoneal injections of GM-CSF in mice had lesser effects on circulating white cell levels but strongly elevated peritoneal macrophage numbers and proliferative activity⁷⁷. Subcutaneous injections of murine IL3 increased bone marrow cellularity and particularly the numbers of mast cells in various tissues^{78,79}. It was also evident that GM-CSF and IL3 injections in mice increased the phagocytic activity of mature macrophages for antibody-coated erythrocytes^{77,78}.

An obvious question to pose was whether CSF injections could enhance resistance in mice to serious fungal or bacterial infections of the type encountered in cancer patients following chemotherapy. This question was examined in mice at the time of the earliest clinical trials on CSFs. In particular, G-CSF injections were tested in multiple infectious disease models, and found to clearly enhance resistance to and survival from a variety of infectious organisms^{80–84}. An important conclusion from these studies was that CSF administration prior to challenge with infectious agents was highly effective, whereas if CSF was administered after infections were initiated, the protective effects were minimal and were only significant if combined with antibiotics.

Do excessive levels of CSF induce toxic effects in mice?

Excess GM-CSF levels either in transgenic mice or in mice repopulated by marrow cells engineered to overexpress GM-CSF, caused excess numbers of granulocytes and macrophages to develop and induced a variety of fatal inflammatory lesions in the lung, muscles, bowel and peritoneal cavity^{85,86}. Similarly, in mice repopulated by marrow cells expressing excess IL3 levels, hyperproliferation of haematopoietic and mast cell populations occurred. This was associated with uncontrollable itching and scratching probably because of mast cell degranulation in the skin⁸⁷. Although repopulation of mice with marrow cells expressing excess levels of G-CSF induced excessive granulopoiesis and very high granulocyte levels, these caused no apparent tissue damage⁸⁸. In subsequent studies, this was radically altered by knocking out the suppressor of cytokine signaling 3 (*Socs3*) gene. SOCS3 is one of the SOCS family of cytoplasmic suppressors of cytokine-initiated receptor signalling and suppresses signalling from activated G-CSF receptors⁸⁹. Mice lacking *Socs3* are hyper-responsive to G-CSF and administration of even normal doses of G-CSF caused hind limb paralysis and death within days from massive accumulations of neutrophils in the spinal cord, liver, lungs and marrow⁹⁰. The lack of toxicity of G-CSF in mice and presumably humans is therefore dependent on the modulating effects of SOCS3.

CSFs and myeloid leukaemia

From studies in the early 1970s on the clonal culture of primary human myeloid leukaemic cells, it was established that chronic myeloid leukaemia (CML) cells formed large, apparently normal, granulocyte or granulocyte-macrophage colonies *in vitro*⁹¹. In contrast, acute myeloid leukaemia (AML) cells often failed to proliferate or at best formed small clusters of progeny *in vitro*⁹². The striking observation was that all CML and most AML cells remained wholly dependent on stimulation by CSF-containing material for

proliferation *in vitro*, the concentrations of CSF required being unexceptional. This situation did not change when purified CSFs later became available⁹³.

This suggested that the CSFs, at a minimum, might be co-factors in the development of myeloid leukaemia, if for no other reason than that they could supply the proliferative and survival stimuli for the clonal expansion of emerging leukaemia cells *in vivo*.

Do sustained excess levels of CSF lead to myeloid leukaemia development? This question has only been posed for GM-CSF. While lifelong excess GM-CSF levels in transgenic mice were not leukaemogenic⁹⁴, these mice were more susceptible to leukaemic transformation by the Moloney leukaemia virus⁹⁵. Repopulation of mice for a period of a few months with marrow cells engineered to produce excess levels of G-CSF or IL3 did not lead to leukaemia development^{87,88}. On this basis, the original simple hypothesis of leukaemia development that led to the search for the CSFs, seemed not to be sustained.

Despite these negative data, at least *GM-CSF* and *IL3* can function as oncogenes in haematopoietic cells. In the initial study, GM-CSF cDNA was inserted *in vitro* into FDC-P1 cells, which is a mouse immortalised haematopoietic cell line. These cells have remained CSF-dependent in culture for the past 25 years and remain non-leukaemic. However, after transfection with GM-CSF cDNA, the FDC-P1 cells were immediately transformed to cells that showed factor-independent growth *in vitro* and behaved as leukaemic cells when transplanted *in vivo*⁹⁶.

In a related series of studies, non-leukaemic FDC-P1 cells were injected into pre-irradiated recipients. The injected cells had therefore not themselves been subject to irradiation but were in a host better able to support the survival of these factor-dependent cells. The cells remained dormant for up to 1 year but eventually most mice developed leukaemia⁹⁷. In each case the leukaemic cells were derived from injected FDC-P1 cells that had acquired the autocrine capacity to produce either GM-CSF or IL3⁹⁸. Analyses showed that this autocrine capacity to produce CSF was determined by the activating insertion of intracisternal A particles in variable locations upstream of one or other CSF gene⁹⁸. It is unresolved why extrinsically applied CSF in high concentrations does not transform immortalized cells such as FDC-P1 after decades in culture, whereas autocrine production of the same CSF leads to immediate transformation. However, the drawback to the FDC-P1 cell experiments was that the molecular basis of the original immortalization of this cell line was never clearly established.

In a more informative experiment, normal bone marrow cells were co-transfected with homeobox B8 (*HOXB8*, also known as *HOX-2.4*) and *IL3* cDNAs. *HOX-2.4* modulates self-renewal and again, there was immediate transformation of the transfected cells to growth factor independence *in vitro* and to leukaemogenicity *in vivo*⁹⁹. This has given rise to the concept that, at least in mice, myeloid leukaemia development requires two types of change: an imbalance of lineage commitment at cell division favouring self-generation or immortalization in extreme form, and the acquisition of a capacity for autocrine growth stimulation.

There is no reason at present to suppose that myeloid leukaemia development in humans differs in principle from that in the mouse. The various leukaemia-associated genes affected by translocation or mutation that have been detected in one or other type of AML presumably achieve one or other of the two changes needed in leukaemogenesis¹⁰⁰. Autocrine production of GM-CSF has been reported in some cases of AML¹⁰¹. However, what is of interest in view of the murine data is that autocrine production of CSF by myeloid leukaemic cells does not appear to be as common in humans as it is in the mouse. It has been reported that, in early CML development, transient autocrine production of IL3 and G-CSF

occurs¹⁰² and the presence of activating mutations in the transmembrane for the extracellular domains of CSF receptors remains a possibility in some AML populations¹⁰³. More commonly, however, autocrine proliferative stimulation in human AML seems to be achieved by other mechanisms, such as activation of cellular *MYC* or *Ras* genes.

A curious outcome of the ability of CSFs to enforce maturation in responding haematopoietic cells is seen in the action of CSFs on some myeloid leukaemia populations. The purification of murine G-CSF was originally monitored using in part an assay system in which proliferation of WEHI-3B myelomonocytic leukaemia cells was suppressed by G-CSF enforced maturation¹³. A dramatic example of this type of action was also observed with the murine GB-2 leukaemia cell line. When these undifferentiated cells were cultured in agar with various CSFs they formed well-differentiated colonies with the correct maturation pattern for the CSFs used⁴². Finally, it could be argued that when CSFs are used to stimulate the growth of human CML colonies *in vitro* which then show normal maturation, the CSF has induced this normal maturation. Unfortunately, responses to the maturation action of CSFs appear to be an uncommon feature of human AML populations and little evidence for a similar therapeutic action of CSFs has been observed in patients with AML receiving injections of CSF.

Clinical trials of CSFs and therapeutic applications

Results of the first clinical trials of CSFs were published in 1988 and 1989. In general these tests were performed following chemotherapy. Similar haematopoietic responses to those in the mouse were noted in these preliminary clinical trials on CSFs in so far as these could be monitored in the peripheral blood and marrow^{104–107}. G-CSF injections elicited clear dose-responsive elevations of blood neutrophil levels and GM-CSF elicited somewhat smaller responses. Responses were maintained for as long as CSF injections were continued and from extended studies on children with abnormally low blood neutrophil levels, no loss of responsiveness was noted after repeated daily G-CSF injections^{108,109}.

Based on these responses and the minimal toxicity associated with the injection of G-CSF or GM-CSF, the licensing of these agents for clinical research was prompt. For example, G-CSF was registered in the US in 1991 for use in the prophylaxis of febrile neutropenia in cancer patients following chemotherapy. Registration of both agents followed in other countries and the indications for clinical use were progressively widened. A less favourable outcome followed trials of M-CSF and IL3. Intravenous infusion of M-CSF in metastatic cancer patients was associated with a fall in platelet levels possibly due to macrophage activation¹¹⁰. Similarly, subcutaneous injections of IL3 in some relapsed lung cancer patients after chemotherapy did increase neutrophil levels but also led to adverse responses, some of which may have been due to mast cell activation¹¹¹. Neither agent has entered clinical use because of the risk of unacceptable side-effects.

Leaving aside the possible special role that CSFs may have in the biology of myeloid leukaemia, the CSFs have had a major impact on the treatment of cancer in two situations: cytotoxic drug-induced neutropenia, and the common need to replace aplastic bone marrow with transplanted haematopoietic cells.

Treating chemotherapy-induced neutropenia

The most common complication of the treatment of cancer with chemotherapy is the development of neutropenia due to bone marrow damage. Low neutrophil levels are associated with a heightened risk of infection¹¹² and a substantial proportion (60%) of patients with febrile neutropenia syndrome (low neutrophil levels plus fever) develop infections. This usually requires hospitalisation and intensive antibiotic therapy. Perhaps of

more importance for those patients where the chemotherapy is potentially curative, episodes of neutropenia, with or without infections, disrupt scheduled chemotherapy resulting in either dose reduction or loss of treatment cycles.

The initial clinical trials of subcutaneously-injected recombinant human G-CSF and GM-CSF were in a miscellany of cancer patients following chemotherapy and the results showed that administration of either agent could elevate neutrophil levels even after chemotherapy^{104–107}. This resulted in a reduction in the duration and severity of the chemotherapy-induced neutropenia (Fig. 1c). In subsequent trials, it was documented in patients with small cell lung cancer and non-Hodgkin's lymphoma that the use of G-CSF or GM-CSF reduced episodes of drug reduction and the frequency of infections^{113–117}. Analysis showed that use of G-CSF allowed patients with chemotherapy-sensitive cancers, such as non-Hodgkin's lymphoma or early stage breast cancer, to avoid dose reductions or delays in their chemotherapy and confirmed that this had an impact on patient survival¹¹⁸. GM-CSF was also used effectively to enhance haematopoietic regeneration after bone marrow transplantation^{119,120} and was initially licensed for clinical use for this purpose.

To date, approximately nine million patients have received G-CSF therapy. These were most often cancer patients in whom cytotoxic drugs had been used. Overall, meta-analyses of multiple controlled trials involving G-CSF have found that G-CSF reduces febrile neutropenia by 46%, the risk of infection-related mortality by 45% and the risk of early mortality from all causes by 40%¹¹⁸. Toxic side-effects of G-CSF have been minor, most commonly, slowly developing bone pain as marrow populations expand¹¹⁸. This may be related to the recent report that sensory nerves have receptors for G-CSF and GM-CSF and that both CSFs can sensitise these nerves to mechanical stimuli¹²¹.

Experience with the use of GM-CSF has been similar, if less extensive than with G-CSF. In the context of providing supporting treatment for patients on chemotherapy, clinical attention has properly been focussed on responding neutrophil levels because of the landmark study linking low neutrophil levels with infections¹¹² and here G-CSF has a stronger action than has GM-CSF. The subtle differences in biological actions between G-CSF and GM-CSF, such as the special actions of GM-CSF on macrophages and dendritic cells, have not yet had much impact on the manner in which the two agents are used clinically.

A notable advance in the use of CSFs for post-chemotherapy neutropenia, was the development of polyethylene glycol-conjugated G-CSF (pegylated G-CSF, pegfilgrastim), which was approved for clinical use in 2002 following Phase II trials on cancer patients receiving chemotherapy^{122,123}. The biological actions of this modified CSF are similar to those of G-CSF, but the larger size of the pegylated molecule prevents renal clearance and greatly increases the life-span of the molecule, a single injection of pegylated G-CSF being the equivalent of a series of daily injections of G-CSF. Studies have shown the efficacy of pegylated CSF in allowing full dose chemotherapy, particularly in elderly patients who would otherwise have been restricted to less toxic, mild to moderate chemotherapy¹¹⁸.

Currently, international guidelines recommend the use of G-CSF as primary prophylaxis when there is an increased risk of febrile neutropenia of greater than or equal to 20%, although the broader use of prophylactic CSF has been suggested^{118,124}. How extensively a non-toxic agent is used is based in part on economic criteria and, when the costs of CSFs are reduced with the introduction of generic CSFs, the occasions on which CSFs may usefully be employed should increase.

From the biological point of view, current clinical practice is probably suboptimal because it has made no use of the powerful synergies to be obtained by combining CSFs or CSFs with

other agents; and has made insufficient use of the fact that CSFs function best when used prophylactically before infections initiate and on bone marrow with reasonable cellularity.

As an aside, and for completeness, there are, of course, less common types of non-cancer patients, such as those with chronic neutropenia or cyclic neutropenia, where the use of G-CSF has been highly effective in preventing infections and has been administered for years without loss of activity or major adverse long-term effects^{125,126}.

It is of interest how the development of the CSFs paralleled that of erythropoietin (EPO), the corresponding regulator of erythroid cell populations. The existence of EPO was recognised long before that of the CSFs but, even so, EPO was purified in 1977¹²⁷, cloned in 1985¹²⁸ and was approved for clinical use in 1989. It is now used routinely in patients with anaemia associated with chronic renal disease and often in cancer patients with anaemia – in both situations to reduce the number of blood transfusions and increase survival and the quality of life^{129,130}. In both types of patient, to avoid cardiovascular complications, EPO-induced rises in haemoglobin levels need to be restricted to below 120g/l.

Haematopoietic Transplantation

During the first clinical trials of G-CSF in cancer patients in 1988 an unexpected observation was made that the patients developed a 100-fold rise in the frequency of colony-forming progenitor cells in the peripheral blood (Fig. 1b)¹³¹. Rises in haematopoietic progenitor cell numbers were also noted in subjects injected with GM-CSF^{132,133}. Although slightly delayed compared with the rises in mature neutrophil levels, the rises were of such a magnitude that it became an intriguing possibility that CSF-induced cells in the peripheral blood might be used for transplantation in place of harvested bone marrow cells.

Subsequent studies in mice in 1990 showed that G-CSF could also elicit rises in haematopoietic stem cells in the blood¹³⁴. With this supporting information, clinical trials were initiated using peripheral blood cells harvested after injections of GM-CSF or G-CSF. Both types of peripheral blood stem cells (PBSC) achieved successful haematopoietic engraftment^{135,136}.

The PBSCs generated were found to result in more rapid rates of restoration of peripheral blood neutrophil levels than those achieved by harvested bone marrow cells and to equal the recovery of cells in patients receiving marrow plus CSF¹³⁷. In addition, CSF-induced PBSCs unexpectedly allowed a more rapid recovery of platelet levels (Fig. 1d)^{136,138}. Thrombocytopenia, requiring treatment with platelet transfusions, is an important reason for continued hospitalisation of cancer patients with myelosuppression following chemotherapy. It is now accepted that the superiority of CSF-elicited PBSCs in transplantation is probably due to the ability to harvest higher numbers of stem and CD34⁺ progenitor cells than by routine bone marrow aspiration. Chemotherapy itself can elevate PBSCs¹³⁹ and higher levels of harvested PBSCs can be obtained by combining CSF with chemotherapy. However, CSF alone usually achieves satisfactory yields of PBSCs and chemotherapy cannot be used when normal donors are providing PBSCs for allografting to patients.

The high cell yields possible after daily injection of CSF or a single injection of pegylated CSF are of great importance when low or medium intensity chemotherapy is used in the treatment of elderly patients. High numbers of haematopoietic progenitor and stem cells are required in these patients to effect adequate engraftment. This is not because of any failure to empty bone marrow niches in the recipient with the less severe chemotherapy. Populations of grafted cells with stable chimaerism can be achieved in normal mice simply by increasing the numbers of transplanted cells to a significant fraction of the resident cells in the recipient¹⁴⁰. This same principle appears to be operative in elderly patients receiving

low dose chemotherapy when the high numbers of harvested PBSCs allow adequate engraftment.

CSF-mobilised PBSCs have now become the dominant cell populations used in transplantations to cancer and other patients. Particularly for normal donors of PBSCs, the safety of G-CSF and GM-CSF is a matter of importance. Extensive clinical experience has shown that CSF induction is a safe procedure without any immediate or long-term consequences¹⁴¹.

Immunotherapy

Dendritic cells are key cellular components of immune responses because of their capacity to capture, process and present antigens to initiate responses in T lymphocytes. GM-CSF was observed to be a major regulator of dendritic cell development *in vitro*^{142–145}, and this has led to study of the positive influence of GM-CSF on immune responses. With the increasing availability of tumour-specific peptides, there has been much interest in the possibility that GM-CSF might be able to enhance specific immune responses against tumour cells. The cancers most frequently considered in this context are melanomas and cancers of the kidney, lung and prostate – cancers for which some evidence exists that host responses can occasionally have significant anti-tumour effects. GM-CSF has variously been co-injected with tumour peptides, injected as a GM-CSF-peptide complex or transfected into sterilised autologous or similar tumour cells using retroviral or adenoviral vectors¹⁴⁶. GM-CSF proved to be the most potent of ten candidate gene products in tests to detect enhanced responses elicited by transfected tumour cells¹⁴⁷. Evidence of enhanced local immune responses within tumours has been obtained in a proportion of patients^{148–154}. To date, positive clinical responses have been restricted to a small subset of injected patients and it is unclear whether these have been superior to those obtained with tumour peptides alone^{146,155}. These are ongoing studies and it is too early to decide whether the use of GM-CSF in immunisation strategies will prove of clinical value.

Conclusions

It has been a long journey since the first agar cultures of marrow cells in 1966. The CSFs have emerged as key regulators of major haematopoietic lineages and two have been in clinical use for two decades to stimulate neutrophil and macrophage production and function, particularly in cancer patients. The use of CSFs to elicit peripheral blood stem cells has revolutionised haematopoietic transplantation, making it simpler, more efficient and more widely applicable in the clinic. Despite this progress, it is still early days in the clinical exploitation of the CSFs to further manipulate haematopoiesis to improve the management of cancer patients.

Although autocrine production of CSF can be involved as one of the steps in the development of myeloid leukaemia, the maturation-inducing effects of CSFs can, conversely, suppress some myeloid leukaemia populations. The clinical application of this complex biology again awaits future developments.

Box 1 **The Road Map of Haematopoiesis****1. Stratified Hierarchy of Haematopoiesis**

Three sequential classes of increasingly numerous ancestors exist in the bone marrow that generate maturing blood cells²⁸. A major separation occurs into cells committed to the formation of myeloid cells and those committed to the formation of T and B lymphocytes. Dendritic cells can be derived from both groups¹⁵⁷. Cells committed to one or other group can have their lineage commitment switched artificially by overexpression of genes such as GATA-1 or PU.1¹⁵⁸.

2. Responsiveness to Regulators

Committed myeloid progenitor cells and their progeny can respond to a single CSF regulator but proliferation is enhanced synergistically by combining regulators. Less mature precursors require costimulation by multiple regulators²⁸.

3. Common Ancestors

Many granulocyte and macrophage precursors have common ancestral cells as do many erythroid and megakaryocyte precursors.

4. Heterogeneity of Individual Cells

Within each maturation category of granulocytes and macrophages there is wide heterogeneity between individual cells in quantitative responsiveness to CSF stimulation and some cells respond better or only to one particular CSF²⁸.

Box 2**① Proliferative stimulation** **② Suppression of apoptosis** **③ Differentiation commitment** **④ Maturation induction** **⑤ Functional stimulation of mature cells**

Operating through a single type of membrane receptor on responding cells, the CSFs are able to elicit a surprising range of biological responses. They are necessary to initiate in a dose-responsive manner every cell division in responding cells²⁸. They prevent cell death from apoptosis^{36–39}. They can, arguably, initiate lineage-commitment and maturation in appropriate haematopoietic subpopulations^{42–44}. Finally, they have powerful effects on the survival and functional activity of mature cells^{47–51}. These pleiotropic effects are made possible by distinct regions of CSF-activated cytoplasmic domains in the receptors^{52–59}.

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Glossary of Terms

Conditioned medium	medium harvested after incubation of cultured cells or tissues.
Transformed	usually indicating an irreversible change from normal to neoplastic cells
Lineage	a sub family of one type of haematopoietic cell ranging from immature to mature cells.
Commitment	the change, usually, irreversible, when a multipotential cell generates or becomes a cell expressing membrane markers and a gene programme restricting the cell to a particular lineage.
Maturation	the sequence of morphological and biochemical changes during which immature cells generate or become mature cells.
Synergy	enhanced cellular responses when two or more regulators interact on target cells.
SOCS family	a family of cytokine (regulator)-induced cytoplasmic inhibitors of signalling from regulator-activated membrane receptors.
Immortalization	a change rendering cells capable of proliferation for prolonged (perhaps unlimited) time periods, the cells usually not being neoplastic.
Neutropenia	abnormally low blood neutrophil levels.
Thrombocytopenia	abnormally low blood platelet levels.
Aplasia	severely reduced cellular content.

Figure 1. The biological actions of the CSFs

(a) CSF-stimulated colony formation *in vitro* by lineage-committed progenitor cells in mouse bone marrow, the sigmoid dose response indicating heterogeneity in the responsiveness of the progenitor cells. Adapted from Fig. 7.1, page 111 of REF. 28; (b) In man, injected G-CSF not only increases peripheral blood neutrophil levels but increases peripheral blood progenitor cells 100-fold. Data shown are from two patients injected with 10 μ g/Kg G-CSF daily for 7 days. Adapted from Fig. 5, page 4443 of REF. 156; (c) In transplanted cancer patients, the injection of G-CSF accelerates the recovery of neutrophil levels following chemotherapy, allowing a shorter duration of hospitalisation. Adapted from Fig. 1, page 893 of REF. 159; (d) Transplantation of CSF-mobilised peripheral blood stem cells augments the rate of recovery of platelet levels following chemotherapy compared with control patients receiving marrow transplants. Adapted from Fig. 4, page 643 of REF. 136.

Timeline Table