41. Villeval JL, Duhrsen U, Morstyn G, Metcalf D. Effect of recombinant human granulocyte-macrophage colony stimulating factor on progenitor cells in patients with advanced malignancies. *Br J Haematol* 1990, 74, 36-44.
42. Siena S, Bregni M, Ravagnani F, *et al.* Heterogeneity of circulating hematopoietic progenitors in cancer patients treated with high-dose cyclophosphamide and recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF). *Haematologica* 1990, 75 (Suppl. 1), 6-10.
43. Ravagnani F, Siena S, Bregni M, Sciorelli G, Gianni AM, Pellegris G. Large-scale collection of circulating haematopoietic progenitors in cancer patients treated with high-dose cyclophosphamide and recombinant human GM-CSF. *Eur J Cancer* 1990, 26, 562-564.
44. Ravagnani F, Bregni M, Siena S, Sciorelli G, Gianni A, Pellegris G. Role of recombinant human granulocyte-macrophage colony stimulating factor for large scale collection of peripheral blood stem cells for autologous transplantation. *Haematologica* 1990, 75 (Suppl. 1), 22-25.
45. Gianni AM, Bregni M, Siena S, *et al.* Recombinant human granulocyte-macrophage colony-stimulating factor reduces hematologic toxicity and widens clinical applicability of high-dose cyclophosphamide treatment in breast cancer and non-Hodgkin's lymphoma. *J Clin Oncol* 1990, 8, 768-778.
46. Gianni AM, Bregni M, Siena S, *et al.* Rapid and complete hemopoietic reconstitution following combined transplantation of autologous blood and bone marrow cells. A changing role for high dose chemoradiotherapy. *Hematol Oncol* 1989, 7, 139-148.
47. Gianni AM, Siena S, Bregni M, *et al.* Granulocyte-macrophage colony-stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. *Lancet* 1989, 2, 580-585.
48. Gianni AM, Bregni M, Siena S, *et al.* Very rapid and complete hematopoietic reconstitution following combined transplantation of autologous bone marrow and GM-CSF-exposed stem cells. *Bone Marrow Transplant* 1989, 4S, 78.
49. Peters WP, Kurtzberg I, Kirkpatrick G, *et al.* GM-CSF primed peripheral blood progenitor cells (PBPC) coupled with autologous bone marrow transplantation (ABMT) will eliminate absolute leukopenia following high dose chemotherapy (HDC). *Blood* 1989, 74, 50.
50. Korbling M, Holle R, Haas R, *et al.* Autologous blood stem-cell transplantation in patients with advanced Hodgkin's disease and prior radiation to the pelvic site. *J Clin Oncol* 1990, 8, 978-985.
51. Pileri A, Tarella C, Bregni M, *et al.* GM-CSF-exposed peripheral blood progenitors as sole source of stem cells for autologous transplantation in two patients with mutiple myeloma. *Haematologica* 1990, 1 (Suppl. 1), 79-82.
52. Elias A, Ayash L, Anderson K, *et al.* GM-CSF mobilized peripheral blood progenitor-cell support after high dose chemotherapy for breast cancer: effect of GM-CSF post reinfusion. *Blood* 1991, 78, 400a.
53. Mazanet R, Elias A, Hunt M, *et al.* Peripheral blood progenitor cells (PBPCs) added to bone marrow (BM) for hemopoietic rescue following high dose chemotherapy for solid tumours reduces morbidity and length of hospitalization. *Proc Am Soc Clin Oncol* 1991, 10, 324.

Eur J Cancer, Vol. 29A, Suppl. 3, pp. S6-S9, 1993
Printed in Great Britain

0964-1947/93 $6.00 + 0.00

Synergistic Interactions in Haemopoiesis: Biological Implications and Clinical Use

T. M. Dexter

Growth factors promote the survival and proliferation of haemopoietic stem and progenitor cells, and in their absence the haemopoietic cells undergo apoptosis and die. The results of studies reported here indicate that multipotent stem cells have receptors for most, if not all, of the growth factors, but that even saturated binding of the receptors for a single growth factor is not sufficient to transduce an effective stimulus for the proliferation of these cells (possibly due to very low receptor numbers). However, when the growth factors are combined synergistic effects can be seen. Studies in which stem cell factor was used in combination with other growth factors showed that stem cell factor allowed the survival of stem cells, while a second growth factor (granulocyte-macrophage colony-stimulating factor) stimulated the stem cells to develop normally. Stem cell factor was also shown to alter the dose-response relationships of developing haemopoietic cells for other growth factors.
Eur J Cancer, Vol.29A, No.3, pp.S6-S9, 1993

THE REGULATION OF NORMAL HAEMOPOIETIC CELL DEVELOPMENT IN THE BONE MARROW

ALL MATURE blood cells are derived from a small number of stem cells which, in adults, are resident in the bone marrow [1]. These stem cells undergo proliferation, differentiation and development and with increasing maturation the cells are released into the circulation. Stem cells possess the potential for extensive self-renewal and they can also differentiate to produce progeny that become progressively more restricted in their developmental options and that possess little or no ability to undergo self-renewal. Ultimately, at least nine different types of mature cells are produced: neutrophils, basophils, eosinophils,

Correspondence to: T. M. Dexter, Christie Hospital and Holt Radium Institute, Wilmslow Road, Withington, Manchester, M20 9BX, UK.
Received 3 Dec. 1992; accepted 29 Mar. 1993.

megakaryocytes, T-and B-lymphocytes, erythrocytes, monocytes and osteoclasts [2].

Within the environment of the bone marrow, stem cells and their progeny are exposed to a variety of stimuli, including physical interactions with other cells mediated by specific cell-adhesion molecules; interactions with extracellular matrix molecules such as fibronectin, collagen and proteoglycans, and exposure to growth-stimulatory and growth-inhibitory cytokines. These stimuli work together to regulate self renewal and differentiation of stem cells and the formation of mature blood cells from their progeny.

In the bone marrow, the developing haemopoietic cells are closely associated with the stromal cells, and there is now a considerable body of evidence suggesting that the stromal cells do not just provide a physical matrix but that they have a direct role to play in the proliferation and differentiation of haemopoietic cells [3]. A crucial feature of stromal-cell-mediated haemopoiesis is the requirement for intimate contact between stromal and haemopoietic cells. This suggests that growth factors are not released at appreciable levels in a soluble form, but are 'localised' to the surface of the stromal cells where they exert their effects by cell-to-cell contact. It has been recognised for several years that molecules which make up the extracellular matrix of stromal cells are able to bind growth factors and present them, in a biologically active form, to the target haemopoietic cells [4]. In this respect heparan sulphate proteoglycan has a particularly important role to play [5]. More recently it has been shown that some growth factors, such as stem cell factor (the ligand for the receptor encoded by the c-*kit* proto-oncogene) [6], may exist as proteins that span the stromal cell membrane; thus possessing an extracellular region (receptor-binding site), a transmembrane portion and an intercytoplasmic region. Therefore, the producer cells and the target cells must be close to each other if a response is to be effected. It has been suggested that specific cell-adhesion molecules mediate interactions between stromal cells and haemopoietic cells [7] and that in the absence of cell adhesion haemopoietic cells will be unable to respond to growth factors associated with stromal cells.

A number of different kinds of stromal cells are present in the bone marrow and appear to be associated with specific haemopoietic cell lineages. Therefore, it may be appropriate to regard the stromal cells of the bone marrow as micro-environments where specific haemopoietic progenitor cells are localised (via cell-adhesion molecules) and stimulated to develop (through the production and/or sequestration of growth factors) into mature blood cells.

If haemopoietic cells are cultured in a medium which contains neither stromal cells, or growth factors, these cells begin to die by a mechanism sometimes referred to as programmed cell death or apoptosis. The nuclei show condensation of chromatin and the DNA starts to break down to form classical nucleosomal fragments consisting of multiples of 200 base pairs. Figure 1 shows the results of culturing purified, highly enriched granulocyte-macrophage progenitor cells (GM-CFC) in the absence of stromal cells and growth factors. In the presence of growth factors the cells survive and proliferate, while in the absence of growth factors they undergo apoptosis and die.

It is possible that in normal 'steady-state' haemopoiesis there

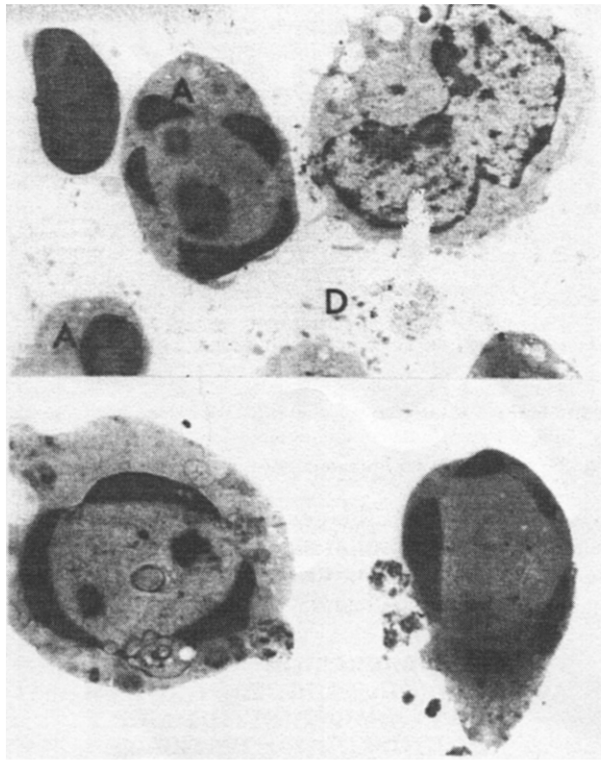

Fig. 1. Granulocyte-macrophage progenitor cells (GM-CFC), cultured in the absence of stromal cells or growth factors, undergo apoptosis and die. This figure shows a normal, healthy cell (top right hand corner) and cells at various stages of apoptosis.

is a degree of overproduction of haemopoietic progenitor cells and that excess cells are destined to die due to limiting concentrations of specific growth factors available in the environment of the bone marrow. Indeed, it may be that these 'redundant' progenitor cells are the ones recruited under the influence of exogenous granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) or erythropoietin to give the excess numbers of circulating blood cells seen in haematologically normal (i.e. 'steady state') patients [8, 9].

TARGET CELLS FOR THE HAEMOPOIETIC CELL GROWTH FACTORS

There is some clear overlap in the biological activities of the haemopoietic cell growth factors, as shown in Fig. 2. Some of the growth factors have many target cells, while others are much more restricted. Interleukin (IL)-3, for example, can stimulate multipotent haemopoietic stem cells (CFC-Mix) to produce clones containing several myeloid cell lineages, and can also promote the development of bipotent and unipotent progenitor cells, leading to the production of the appropriate mature cell types. GM-CSF is also a multilineage-stimulating factor, but it acts only on GM-CFC and eosinophil progenitor cells (Eos-CFC), leading to the development of neutrophils, monocytes and eosinophils. Macrophage colony-stimulating factor (M-CSF) acts upon the GM-CFC, leading to the production (primarily) of monocytes/macrophages. G-CSF and erythropoietin act on the neutrophil and erythroid precursor cell

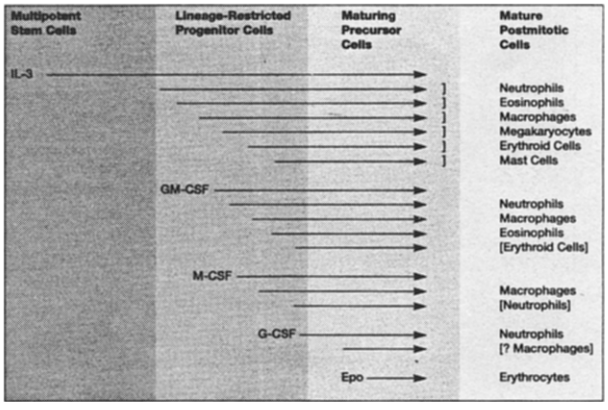

Fig. 2. Target cells for haemopoietic cell growth factors *in vitro*.

populations and are the most lineage-restricted of the haemopoietic cell growth factors [10]. However, within the bone marrow haemopoietic stem cells and their progeny may be exposed to a variety of stimuli at any one time.

HOW DIFFERENT GROWTH FACTORS MAY BE REGULATING THE GROWTH AND DEVELOPMENT OF THE EARLY MULTIPOTENT STEM CELLS

Superficially, the data in the section above appears to suggest that multipotent stem cells have receptors for only one or two of the growth factors (e.g. IL-3), and that the receptors for the other growth factors are acquired during, or perhaps as a consequence of, differentiation. Progenitor cells therefore respond to those growth factors for which they have acquired receptors and which are available in the environment. They then undergo proliferation and development to produce the mature cells appropriate to this pattern of receptor-factor availability. However, the alternative model which should be considered is that the multipotent stem cells possess receptors for most, if not all, the growth factors and that the lineage choice and development is a reflection of the range of growth factors to which the multipotent stem cells are exposed. Normal bone marrow cells are not suitable tools for investigating these models, as they will be 'contaminated' with some T-lymphocytes and macrophages and these can produce cytokines which could influence the response to the primary growth factor which is to be examined in the culture system. Therefore, purified, highly enriched populations of multipotent stem cells (CFC-Mix) and GM-CFC are used. When GM-CFC are cultured with different growth factors, IL-3, GM-CSF and M-CSF, by themselves, are very effective at stimulating colony development. G-CSF is a relatively poor stimulus, since the main targets for this growth factor are the maturing granulocyte precursor cells. When growth factors are used in combination, no synergistic or additive effects are seen, indicating that maximal stimulation of GM-CFC has already been seen using optimal concentrations of single growth factors. However, when multipotent stem cells are cultured a different picture is seen. IL-3, alone, has only a modest colony-stimulating effect, approximately 0.2% of cells forming clones containing mixed myeloid cells [11], whereas stem cells died when they were cultured in GM-CSF, M-CSF, G-CSF or interleukin-1 alone [11]. These data again superficially seem to suggest that stem cells do not have receptors for these other growth factors. However, when growth factors are used in various combinations, they can exert a powerful stimulus, with progressively more multipotent stem cells being recruited into proliferation and colony development [12]. For example, the combination of M-CSF with IL-3 gives four times the number of clones seen with IL-3 alone, and IL-1 with IL-3 gives five times the number of clones seen with IL-3 alone. Of particular interest are the combinations of GM-CSF with G-CSF or M-CSF. None of these factors can promote the survival or growth of multipotent stem cells on their own, but when combined they stimulate the development of at least as many clones as IL-3 alone. This work has two important implications. The first is that in order to get the optimal response from these cells several growth factors may have to be added at once, in appropriate concentrations. When the stimuli are temporally separated, the response decreases with the time interval between addition of the growth factors. The second important feature is that the outcome of the response, in terms of the mature cells produced, is a reflection of the growth factors to which the stem cells are exposed. For example, with IL-3 multiple myeloid cell lineages are produced, whereas with M-CSF plus G-CSF (which recruits the same cell population as that recruited by IL-3), the resulting colonies contain only neutrophils and macrophages [11, 12] (see Fig. 3).

These data indicate that multipotent stem cells do indeed have receptors for most, if not all of the growth factors, but that even saturated binding of the receptors for certain growth factors is not sufficient to transduce an effective stimulus for proliferation of these cells (possibly due to very low receptor numbers). However, when the growth factors are combined, synergistic effects can readily be seen.

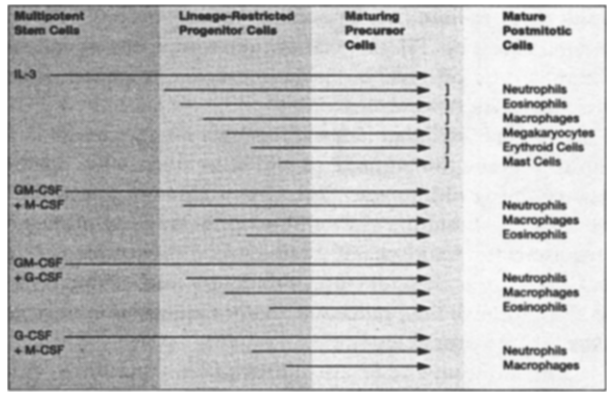

Fig. 3. Target cells for combinations of haemopoietic cell growth factors *in vitro*.

STEM CELL FACTOR

There has been considerable interest recently in stem cell factor (also known as mast cell growth factor or *kit* ligand) and we have investigated its effects on the growth of multipotent stem cells *in vitro* using day 12 CFU-S (murine spleen colony-forming unit cells). As discussed previously (see Fig. 1), it was found that, when used alone, M-CSF, G-CSF and GM-CSF were not directly growth-stimulatory for stem cells. Stem cell

factor alone also did not stimulate proliferation but did allow survival of the stem cells (i.e. it suppressed apoptosis) and when it was combined with agents such as GM-CSF the cells began to proliferate and develop into mature cells. Furthermore, the combination of stem cell factor plus GM-CSF gave a response almost as great as that seen with IL-3 plus stem cell factor. Thus, this is an example of a combination of one growth factor (stem cell factor) which allows the survival of the stem cells and a second (GM-CSF) which allows the stem cells to proliferate and to proceed through their normal developmental programme.

Stem cell factor has also been shown to alter the dose-response relationships of developing haemopoietic cells for other growth factors. The response of stem cells has been measured in a colony-forming assay to IL-3, either alone or with stem cell factor. The dose of IL-3 was titrated down to a concentration at which no colonies developed and the response, with and without stem cell factor, was recorded at each dose level. When stem cell factor was added, obvious synergy was apparent at the higher doses of IL-3. At doses which did not induce proliferation, the addition of stem cell factor resulted in at least as great a response as that seen with the higher (effective) doses of IL-3 alone. Thus, when considering combinations of growth factors for use *in vivo* the concentrations used should also be taken into account.

The following study was carried out to see if the alteration in the dose-response relationship observed with stem cell factor *in vitro* is also seen *in vivo*. G-CSF was administered to mice at a therapeutically effective dose of 10 μg/kg/day. The dose was then titrated down to 1.0 μg/kg/day and 0.1 μg/kg/day. At the two lower doses no change was seen in the leucocyte count, however, when stem cell factor was administered in addition to G-CSF, the response exceeded the additive effect of the two growth factors [13]. The combination of stem cell factor plus the highest dose of G-CSF yielded leucocyte counts of up to around 250 million per millilitre (see Fig. 4) and in addition the lowest dose of G-CSF (0.1 μg/kg/day), which is only one hundredth of the normal therapeutically effective dose, when given with stem cell factor produced responses at least as great as those seen with doses of 10 μg/kg/day G-CSF alone. This shows that stem cell factor acts in synergy with G-CSF *in vitro* and indicates that similar effects may well occur with a combination of stem cell factor plus GM-CSF.

There are now possibilities, therefore, not only for combining cytokines *in vivo*, but also for reducing their doses depending on the combination being used. The therapeutic implications of these findings are obvious and extensive phase I/II clinical trials, currently being carried out, should provide the information from which novel treatment strategies can be developed.

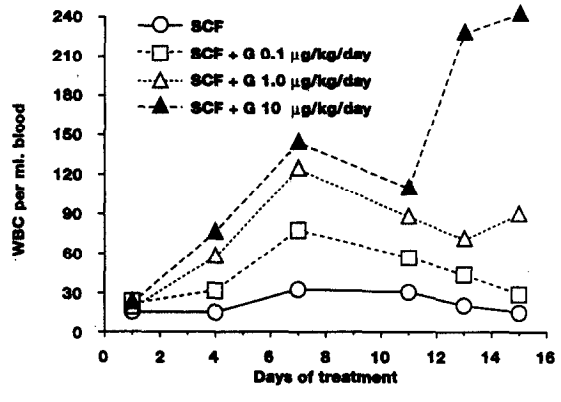

Fig. 4. The changes in peripheral blood nucleated cell count in mice during treatment with stem cell factor (SCF), 100 μg/kg/day, either alone or in combination with G-CSF (G) at doses of 0.1, 1.0 and 10 μg/kg/day. Adapted with permission from Molineux *et al.* *Blood*, 1991, 78, 961-966. W.B. Saunders Co. [13].

1. Metcalf D, Moore MAS. In Haemopoietic Cells, North Holland, Amsterdam, Holland. 1971.
2. Dexter TM. Haemopoietic growth factors. *Br Med Bull* 1989, 45, 337-349.
3. Dexter TM. Stromal cell associated haemopoiesis. *J Cell Physiol* 1982, 1, 87-94.
4. Gordon MY, Riley GP, Watt SM, Greaves MF. Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature* (London) 1987, **326**, 403-405.
5. Roberts RA, Gallagher JT, Spooncer E, Allen TD, Bloomfield F, Dexter TM. Heparan sulphate bound growth factor: A mechanism for stromal cell mediated haemopoiesis. *Nature (London)* 1988, 332, 376-378.
6. Witte ON. Steel locus defines new multipotent growth factor. *Cell (Cambridge, Mass)* 1990, **63**, 5-6.
7. Kincade PW. The lymphopoietic microenvironment in bone marrow. *Adv Cancer Res* 1990, **54**, 235-273.
8. Lord BI, Gurney H, Chang J, Thatcher N, Crowther D, Dexter TM. Haemopoietic cell kinetics in humans treated with rGM-CSF. *Int J Cancer* 1992, **50**, 26-31.
9. Lord BI, Bronchud MH, Owens S, *et al.* The kinetics of human granulopoiesis following treatment with granulocyte colony stimulating factor *in vivo*. *Proc Nat Acad Sci (Wash)* 1989, **86**, 9499-9503.
10. Whetton AD, Dexter TM. Myeloid haemopoeitic growth factors. *Biochim Biophys Acta* 1989, **989**, 111-132.
11. Heyworth CM, Ponting ILO, Dexter TM. The response of haemopoietic cells to growth factors: developmental implications of synergistic interactions. *J Cell Sci* 1988, **91**, 239-247.
12. Dexter TM, Heyworth CM, Spooncer E, Ponting ILO. The role of growth factors in self-renewal and differentiation of haemopoietic stem cells. *Philos Trans R Soc (London)* 1990, **327**, 85-98.
13. Molineux G, Migdalska A, Szmitkowski M, Zsebo K, Dexter TM. The effects on hematopoiesis of recombinant stem cell factor (ligand for c-*kit*) administered *in vivo* to mice either alone or in combination with granulocyte colony-stimulating factor. *Blood* 1991, 78, 961-966.