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The molecular control of hematopoiesis

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Experiments both *in vitro* and *in vivo* have increased our understanding of how cytokines can act to influence the maintenance of the primitive hemopoietic populations and the production of mature blood cells. Such understanding helps us to use cytokines as therapeutic agents in order to manipulate the hemopoietic response to disease and its treatment.

Key words: Granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, hemopoietic stem cells, macrophage inflammatory protein-1α, stromal cells, transforming growth factor-β.

Introduction

Within the bone marrow, the hemopoietic stem cells are found in intimate association with a complex stromal cell network and its associated extracellular matrix molecules. This makes up the environment in which stem cells survive, self-renew and undergo differentiation, and in which the more developmentally restricted progeny of the stem cells can develop into mature blood cells prior to their release into the circulation. The hemopoietic growth factors represent part of an integrated network of molecules which operate within the bone marrow to regulate hemopoiesis.

Hemopoiesis takes place in the bone marrow in association with a number of different kinds of stromal cells[1] which produce a variety of growth factors. Some of which are membrane-bound, some are present in soluble form, some exist both in membrane bound and soluble form, and others are released and become bound by the extracellular matrix molecules for presentation to the target cells. These matrix molecules are important for the presentation of growth factors and may prevent some growth factors from undergoing proteolytic digestion.[2] Close contact is required between the producer (stromal) cells and the target (hemopoietic) cells if a response is to be effected. This is achieved through specific cell-adhesion molecules which mediate interactions between the stromal cells and hemopoietic cells (stem cells and their progeny), thus enabling the hemopoietic cells to obtain the stimuli necessary for self-renewal, development and differentiation into mature cells.[3]

It is recognized that growth factors are essential for the survival of target cells and some, though not all, are potent mitogens. However, the questions of whether growth factors are necessary for differentiation to occur and whether proliferation and differentiation need to be coupled remained to be resolved.

Growth factors

Growth factors and survival of target cells

Classical studies in experimental hematology showed that when bone marrow cells were removed from the body and attempts made to culture them in the absence of growth factors, they died. It is now known that cell death occurred through a mechanism involving apoptosis: the cells shrink, the chromatin condenses and DNA breaks down into 200 base pair nucleosomal fragments.[4] Figure 1 shows hemopoietic cells that have been cultured for 18 h in the presence and absence of growth factors. It can be seen that the cells cultured in the absence of growth factors exhibit the diagnostic characteristics of apoptosis described above.[5]

It may be that there is always a modest overproduction of developmentally restricted cells in the bone marrow and in situations where the concentrations of growth factors are limited some of these cells may be destined to die. If this is the case, then some of the effects seen in patients treated with growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF)[6] and granulocyte colony stimulating factor (G-CSF),[7] may be due to these substances acting not only to

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increase proliferation but also as simple survival signals. Thus, they may be allowing cells that would otherwise die to survive and then to proceed through the various maturation stages to produce the mature cells.

Clinical studies carried out by Mertelsmann and Buchner (personal communication) in patients with acute myeloid leukemia showed that treatment with GM-CSF was associated with a dramatic increase in leukemic blast cells and when GM-CSF treatment was stopped the blast cell population then fell to pretreatment levels. The cells died through a mechanism involving apoptosis. So it appears that leukemic cells also require growth factors to survive, which presents interesting possibilities for the exploitation of normal cell death mechanisms as targets for developing chemotherapeutic treatments.

Growth factors and proliferation of target cells

For many years it has been known that if hemopoietic progenitor cells are cultured in a medium containing a low concentration of a growth factor, such as macrophage colony stimulating factor (M-CSF), they will survive but will not proliferate. If the concentration of growth factor is increased then the cells will also undergo proliferation.[8,9] Many of the growth factors are mitogenic as well as being survival signals. However, survival can be uncoupled from proliferation. A growth factor may allow the cells to survive but not proliferate, or it may allow just one or two rounds of proliferation followed by death of the cells.

In addition to inducing the proliferation of cells in patients, in animals and in *in vitro* cultures, growth factors can also alter cell cycle kinetics. A number of investigators have reported that when patients are treated with GM-CSF the proportion of committed granulocyte-macrophage colony forming cells (GM-CFC) which are in an active phase, undergoing DNA synthesis, increases and the same is also true for primitive burst-forming units-erythroid (BFU-E) and colony-forming units-megakaryocyte (CFU-Meg), as illustrated in Table 1. Prior to treatment with GM-CSF, the population of GM-CFCs are already cycling, such that many of these cells are in an active phase and around 40% of them would be killed if exposed to a thymidine suicide treatment regime. So how can GM-CSF cause an increase in the proportion of cells

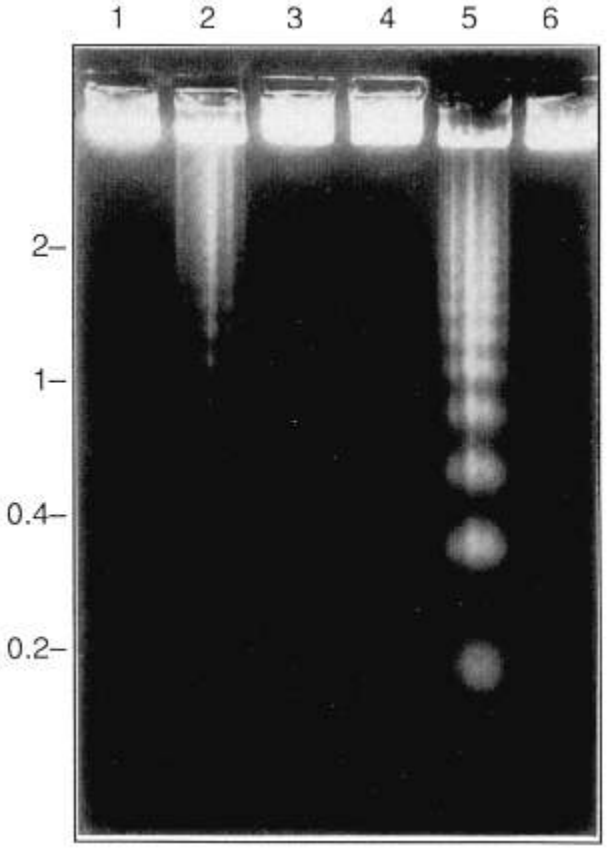

Figure 1a. Degradation of nuclear DNA after growth factor deprivation of dependent cell lines. Lanes 1–3, total DNA from FDCP-mix C2GM cells: lane 1, 0 h; lane 2, 18 h in absence of GM-CSF; lane 3, 18 h in presence of GM-CSF. Lanes 4–6, total DNA from FDCP-mix 1G cells: lane 4, 0 h; lane 5, 18 h in absence of G-CSF; lane 6, 18 h in presence of G-CSF.

undergoing DNA synthesis in an already cycling population? The answer is that it alters the cycling characteristics of these cells. Granulocyte precursor cells normally have a duration of DNA synthesis of 13–14 h; however, with the addition of GM-CSF this is reduced to 7–9 h. Similarly the complete cycle time of these cells is reduced from 70–90 to 21–35 h with GM-CSF. The differential effect between the reduction in DNA synthesis time (approximately 50%) and the total cell cycle time (approximately 70%) is responsible for the increased proportion of progenitor cells undergoing DNA synthesis seen with GM-CSF and ultimately for the increased levels of mature cells which are seen.

A study looking at the production of monocytes and macrophages in response to GM-CSF showed that monocyte counts can indeed be increased in patients in response to GM-CSF (Table 2).[6] The time of peak appearance in the peripheral blood,

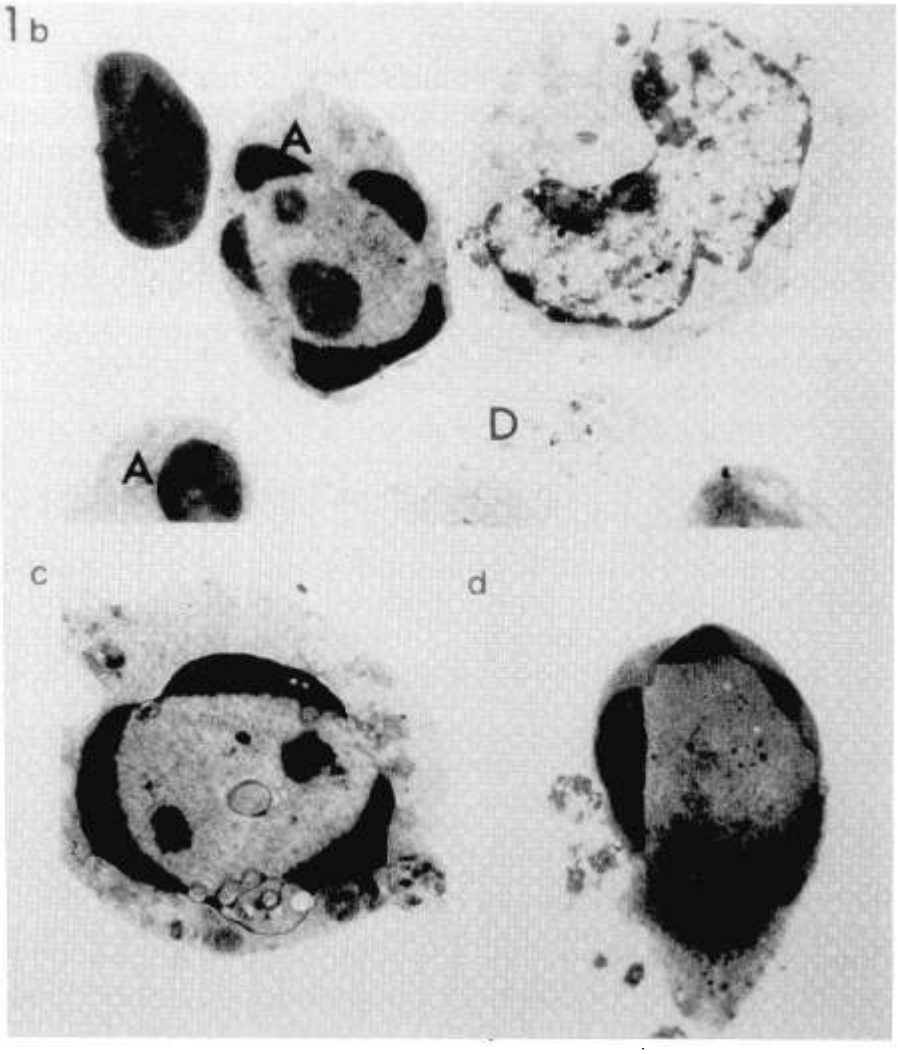

Figure 1b–d. Electron micrographs of growth factor deprived FDCP-mix (b) and FDCP-1 cells (c,d) showing diagnostic condensation of chromatin. Cells undergoing apoptosis are labelled A, and cell debris is labelled D.

Table 1. Proliferative activity of committed hemopoietic progenitor cells before, during and after GM-CSF treatment

	% in DNA synthesis			
	Pre GM-CSF	On GM-CSF	Post GM-CSF	Source (ref.)
14 day GM-CSC	43	82 (3)	44 (4)	16
			42 (7)	16
7 day GM-CFC	41	56 (3)	38 (4)	16
			30 (7)	16
	16	48 (14)	10 (21–28)	17
BFU-E	32	79 (3)	50 (4)	16
			23 (7)	16
	9	58 (14)	3 (21–28)	18
CFU-Meg	31	88 (3)		19

Figures in parenthesis indicate days after start of treatment.

Table 2. Growth factor stimulated monocyte production

	Maximum monocyte count $\times 10^{-4}$/ml	Time of peak appearance in peripheral blood (h)	Peripheral half-life $t_{1/2}$ h	Amplification factor	Extra maturation divisions
Control	32	48	46	1	0
GM-CSF	106	24	31	4.5	2.2

from formation in the bone marrow and release into the blood was halved from 2 days to 1 day, while the peripheral half life of cells being produced was only slightly less than that seen in normal individuals. This increase in monocyte counts represents an amplification factor of about four and a half, which is two extra divisions in the maturation compartment. Two (or even three or four) extra divisions in the maturation compartment is only a modest effect relative to the total capacity of the hemopoietic system, but results in large increases in cell number. The increase in granulocyte production is accounted for by the reserve capacity of the committed progenitor (GM-CFC) pool, with a reduction in cell cycle time and an increase in the proportion of progenitors undergoing DNA synthesis at any time. Agents such as GM-CSF, G-CSF and possibly interleukin (IL)-3 do not appear to affect the most primitive cell population: they do not make the stem cells cycle more and they do not divert the stem cells into one or other of the cell lineages.

As previously mentioned, it has been observed that the proportion of BFU-E and CFU-Meg cells undergoing active DNA synthesis is also increased with GM-CSF treatment (Table 1) and yet no increase in levels of erythrocytes or platelets is seen. Do these cells have receptors for GM-CSF? If yes, then why do they not produce more erythrocytes and platelets? A clue to these questions may be found in the results of studies carried out by Metcalf *et al.*[10] in which it was found that a population of multipotential cells can divide in response to GM-CSF but will not form colonies. The reason for this phenomenon may be that while GM-CSF may stimulate the cells to undergo one or two rounds of DNA synthesis, it does not provide sufficient stimulus to keep the cells alive and they die by a mechanism involving apoptosis.

Thus, it is apparent that for some cells GM-CSF is a survival signal and a mitogen, and for others it is a potent mitogen but not a survival signal. With this in mind, we can begin to understand the effects seen with combinations of growth factors.

The use of combinations of growth factors

When day 12 CFU-S multipotential cells are exposed to IL-3 many colonies are formed after 14 or more days of culture, but when they are exposed to stem cell factor, few or no colonies are seen. This is because stem cell factor is not an adequate stimulus for the cells to proliferate into clones which can be recognized in a soft gel matrix. Similarly, no or very few clones are seen to develop when multipotential cells are exposed only to IL-1, IL-4, IL-6, G-CSF, M-CSF or GM-CSF. However, when stem cell factor is used in combination with other growth factors, such as IL-6, M-CSF, GM-CSF or G-CSF, a tremendous synergy is seen (CM Heyworth, unpublished). For example, when stem cell factor and GM-CSF are combined, almost as many clones of the same cell lineages are produced as when stem cell factor is combined with IL-3. We suggest that this synergistic effect occurs because stem cell factor is a potent survival signal, while GM-CSF is a potent proliferative stimulus, and together they allow the cells to survive and proliferate.

However, when a highly purified population of more developmentally restricted cells, GM-CFC, is used a different pattern of response is seen.[11] The cells respond well in the presence of IL-3, GM-CSF and M-CSF (in the presence or absence of serum for IL-3 and GM-CSF), and respond little or not at all to G-CSF. If stem cell factor is added to IL-3, M-CSF or GM-CSF, only a modest additional effect is seen, but significant synergy is seen when stem cell factor is given in combination with G-CSF (see Figure 2). The synergistic effects are even more marked in serum-deprived cultures. With IL-3 and GM-CSF, stem cell factor produces a modest additional effect, but while M-CSF and G-CSF alone produce few colonies, when stem cell factor is added a synergistic effect is seen (see Figure 2). Thus, under serum-deprived conditions, both G-CSF and M-CSF require stem cell factor for proliferation and further development of clones.

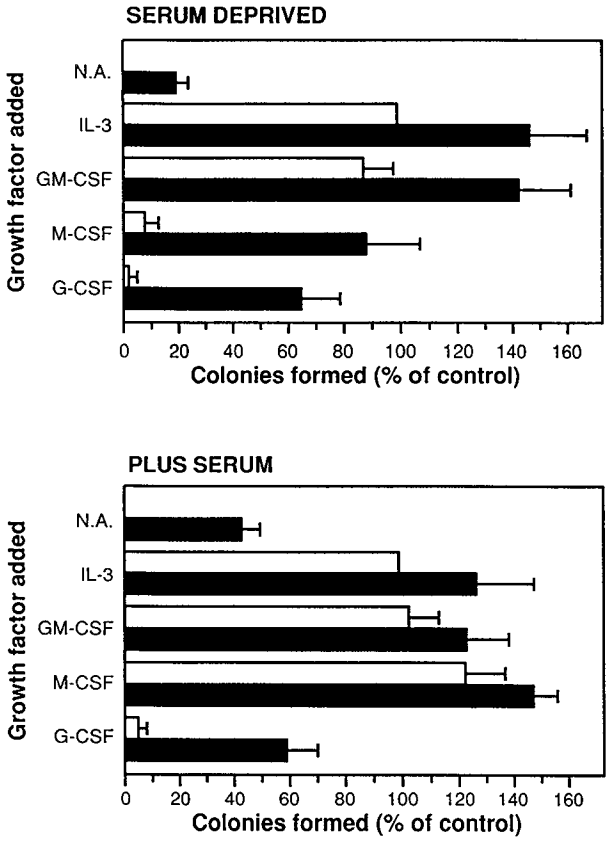

Figure 2. The effect of SCF on colony formation by an enriched GM-CFC population in response to hemopoietic growth factors. Colony formation was assessed in the presence of GM-CSF, IL-3, M-CSF and G-CSF, either alone (□) or in combination with 100 ng/ml SCF (■) in the presence and absence of serum. Results are expressed as a percentage of the colony formation in the presence of 100 U/ml IL-3 alone and represent the means of more than six observations ±S.D.

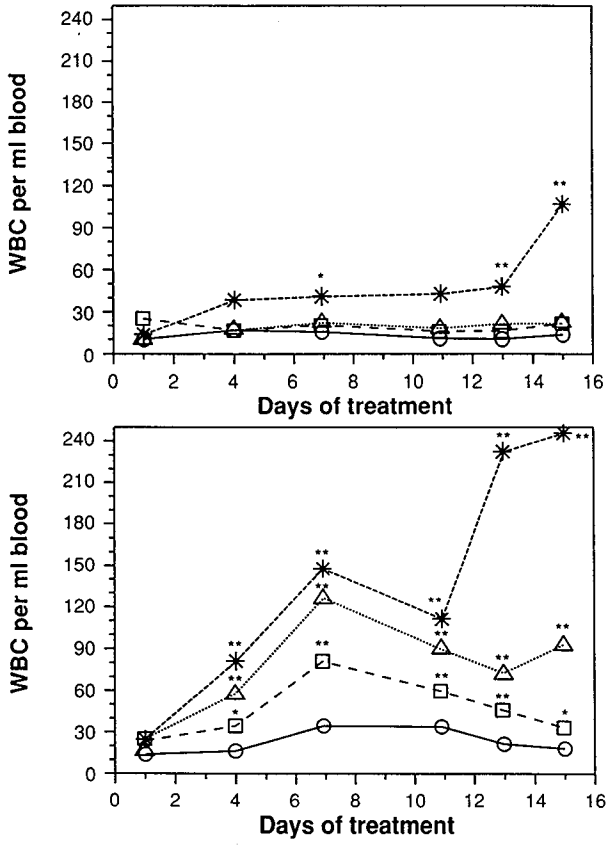

Figure 3. Peripheral blood cell count in mice treated with carrier alone, SCF alone, or various doses of G-CSF (units/kg/day) alone and in combination with SCF (100 μg/kg/day). * Denotes points which differ significantly from control ($p < 0.05$) and ** where $p < 0.01$. —⊖—, PBS; -⊟-, G1,500; ···△···, G15,000; --✳--, G150,000.

On the basis of the findings of these *in vitro* studies, one might well expect to see synergy between G-CSF and stem cell factor *in vivo*. GM-CFC not recruited by G-CSF alone might be recruited with the addition of stem cell factor, resulting in increased production of neutrophils and monocytes. To see whether the synergistic effect seen *in vitro* was also seen *in vivo*, G-CSF was administered to mice at a therapeutically effective dose of 150 000 U/kg/day and the effects compared with those seen at 15 000 and 1500 U/kg/day.[12] The lower dose levels of G-CSF alone produced no increase in the number of circulating neutrophils. However, when stem cell factor was given in combination with G-CSF, the extremely low dose of 1500 U/kg/day G-CSF gave a similar response to that seen with the dose of 150 000 U/kg/day G-CSF given alone (see Figure 3). At the highest dose of G-CSF (150 000 U/kg/day) large responses, in terms of numbers of circulating mature neutrophils and monocytes, were seen, which exceeded the additive effect of the two growth factors.

GM-CSF is both a potent survival signal and a mitogen for GM-CFC, and no synergy is seen when it is given in combination with stem cell factor. However, GM-CSF might require stem cell factor for the recruitment of more primitive cells, such as BFU-E and CFU-Meg. If this is the case, stem cell factor and GM-CSF could be given together for a few days to allow the primitive cells to survive and proliferate, which they could not do if GM-CSF was given alone. Then the stem cell factor could be discontinued and GM-CSF given alone, and an increase in other cell lineages, not normally seen with GM-CSF, might be seen.

Growth inhibitory molecules

Only growth stimulating molecules have been discussed up to this point. However, some growth inhibitory molecules may also have a role to play in integrating the control of proliferation and development of cells.

Transforming growth factor (TGF)-β inhibits the proliferation of multipotent stem cells and of GM-CFC when administered *in vivo* to mice, taking 5 days to exert its maximal effect. On the other hand, macrophage inflammatory protein-1α (MIP-1α), one of a family of products produced by activated macrophages, takes multipotent cells out of cycle and into a quiescent state within a few hours after a single dose. It has been suggested that the lag time seen with TGF-β occurs because, in some cell types, TGF-β appears to modulate the expression of receptors for the growth stimulating molecules, and it must inevitably take some time

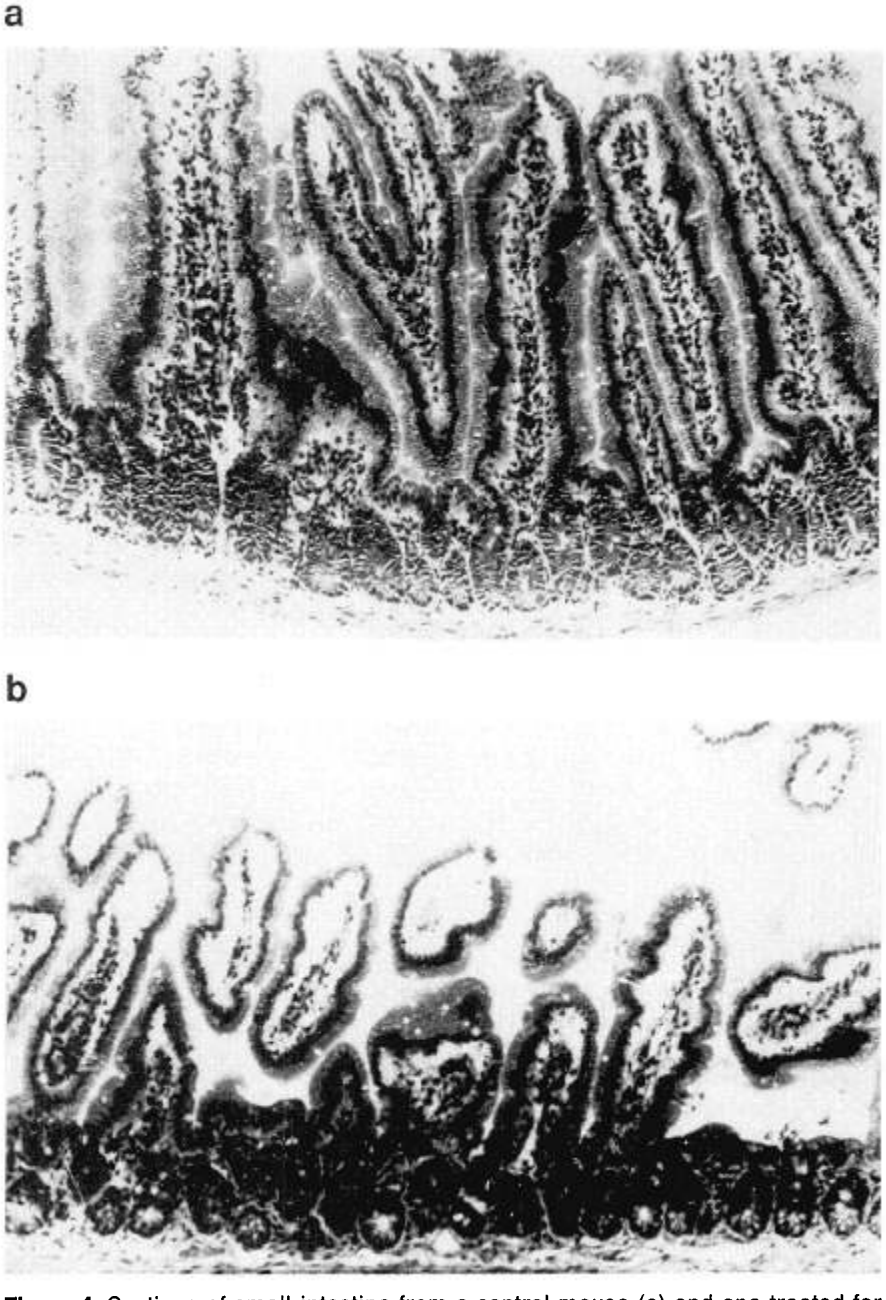

Figure 4. Sections of small intestine from a control mouse (a) and one treated for five consecutive days with 100 μg/kg/day TGF-β (b).

for reduction of the numbers or the affinity of the receptors to occur. Treatment with either TGF-β or MIP-1α causes the cells to come out of the proliferative state, but still survive.

The effects of TGF-β are not restricted to the hemopoietic system. Figure 4 shows the effects in the intestine of animals treated with control solvent and with TGF-β.[13] Effects are seen on the crypt and the villus size within the intestine, indicating an effect of TGF-β on the proliferative compartment within the intestine. It is well known that chemotherapeutic agents do not just kill tumor cells. They also kill normal cells, resulting in a spectrum of side effects, which range from unpleasant or uncomfortable to lethal. Growth factors do not protect the system, they only accelerate the recovery of cells once the damage has been done. The use of agents which may confer protection on the hemopoietic and other regenerating cell systems is an attractive possibility as part of a treatment regime for cancer. For example, such agents could be used to put regenerating normal cell systems out of cycle, while cycle-specific chemotherapeutic agents are used to try to reduce the tumor burden to a critical level.

With this in mind, a model has been developed to see whether MIP-1α can be used to protect normal stem cells from the effects of cycle-specific agents. Hydroxyurea, an agent which is used clinically, kills many of the cycling committed progenitor cells, which are undergoing DNA synthesis but few of the more primitive stem cells when administered as a single injection to mice. The reason for this is that in normal animals (and presumably the same is true in patients) most of the hemopoietic stem cells are essentially quiescent and are unaffected by the treatment. However, the damage to the committed cells is somehow recognized within the system and, as a consequence of this depletion, the multipotent stem cells go into cycle. In mice, cycling happens in a semi-synchronized way and within 7 h the majority of the stem cells will be cycling. Consequently, if a second injection of hydroxyurea is given, the majority of these multipotential cells will be killed. If, however, MIP-1α is given between the first and second doses of hydroxyurea, it will prevent the stem cells from going into cycle and should result in enhanced survival of these cells following the second injection. Figure 5 shows the effects of using MIP-1α in this way.[14] When hydroxyurea was administered to the mice at 0 and 7 h, without MIP-1α, many multipotential stem cells were killed and the recovery curve was recorded (the curve for recovery of day 11 CFU-S

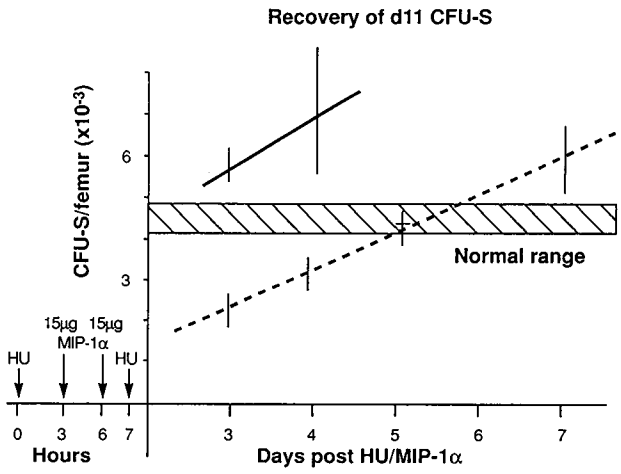

Figure 5. Recovery of day 11 CFU-S in mice treated with 900 mg/kg hydroxyurea (HU) at 0 and 7 h either without MIP-1α treatment (broken line) or with administration of two doses of 15 μg MIP-1α at 3 and 6 h after the first injection of HU (solid line).

between days 4 and 7 following administration of hydroxyurea is shown, as it was not possible to measure the small numbers of these cells from day 1). However, when MIP-1α was given 3 and 6 h after the first dose of hydroxyurea, complete protection of the stem cells was observed. More cells were seen than would be expected even in normal, completely untreated mice, indicating that MIP-1α is not only protecting the cells, but is also preventing them from undergoing differentiation.

An interesting possibility for the clinical application of MIP-1α has arisen from the observation by Eaves *et al.*[15] that the most primitive tumor cells which can be isolated from patients with chronic myeloid leukemia do not respond to MIP-1α. These patients would appear to be suitable subjects for a trial combining MIP-1α with cell cycle specific chemotherapy, in an attempt to control the malignant clones while protecting the patient's normal stem cells.

Conclusions

Growth factors, such as GM-CSF and G-CSF, have a major role to play in the treatment of patients with malignant disease and other hematological disorders: enhancing regeneration and reducing the period of neutropenia following cytotoxic therapy, and for the collection of stem cells from the peripheral blood for autografting.

The use of agents, such as TGF-β and MIP-1α and other molecules currently under investigation,

which reduce the proliferation of normal stem cells in the bone marrow, intestinal tract and skin, may permit the use of high dose intensity regimes of cell cycle-specific chemotherapy, and result in more effective and less toxic treatments.

However, it is unlikely to be possible to prevent damage to all types of cells and therefore the growth factors will still play a key role in post-treatment regeneration of various cell populations. Future research is likely to focus on determining which combinations of growth stimulatory and growth inhibitory factors provide most clinical benefits.

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