Induction of apoptosis in myeloid progenitors by granulocyte–macrophage colony-stimulating factor

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ABSTRACT

Spontaneous apoptosis of normal purified bone marrow $CD34^+$ cells induced by granulocyte–macrophage colony-stimulating factor (GM-CSF) via the Fas pathway appears to be mediated by caspase-1 and caspase-8 activity. In seeking an alternative explanation for this observation, the present study examined $CD34^+$ cell growth with different cytokines, cytokine concentrations, caspase inhibitors, cell crowding and different media. Exposure of the normal $CD34^+$ cells to different concentrations of GM-CSF and granulocyte colony-stimulating factor (G-CSF) increased apoptosis at lower concentrations. However, these GM-CSF effects were suppressed by G-CSF. Investigation of the association between apoptosis and crowding and different media showed that: 1) G-CSF and GM-CSF are equally effective as survival factors, and 2) the percentage of apoptotic cells in liquid culture was markedly lower than that found in methylcellulose culture. Finally, immunofluorescence staining showed that Fas was expressed at 10 ng/mL GM-CSF, while Bcl-2 expression was detected at 100 ng/mL. These findings suggest that cytokine concentration, cell culture conditions, cell crowding and cell interactions all are important factors in GM-CSF-induced apoptosis.

KEY WORDS: Antigens, CD34. Apoptosis. Caspases. Cytokines. Fluorescent antibody technique. Granulocyte-macrophage colony-stimulating factor. In situ nick-end labeling.

Introduction

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is produced by various normal and malignant cells[1] and is the growth factor for many cell types including haemopoietic and non-haemopoietic cells[2]. Endothelial cells, fibroblasts and osteoblast produce GM-CSF after tumour necrosis factor (TNF) stimulation, while T lymphocytes produce it following stimulation with antigen or phytohaemagglutinin (PHA).

Initially, GM-CSF was characterised by its ability to stimulate proliferation of granulocytes and macrophages, although it exerts a direct effect on the growth of megakaryocyte colonies.[3] It acts synergistically with interleukin-3 (IL-3) and erythropoietin (Epo) to increase multipotential and erythroid colony formation, and with macrophage colony-stimulating factor (M-CSF) to induce the growth of macrophages.[4] However, GM-CSF has a role as an apoptosis-inducing factor that remains poorly understood.

This study investigates the possibility that GM-CSF exposure may induce apoptosis during colony growth and examines $CD34^+$ cell growth with different cytokines, cytokine concentrations, caspase inhibitors, cell crowding and different media.

Materials and methods

Reagents
All recombinant human cytokines (Table 1) were obtained from First Link, UK. A caspase-1 protease inhibitor (YVAD) and a caspase-8 specific inhibitor (IETD) were obtained from Bachem and Calbiochem, respectively. Anti-Fas fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (PharMingen, Oxford, UK) was used at 10 µmol/L. Anti-Bcl-2 FITC-conjugated monoclonal antibody (DakoCytomation, UK) was used at a dilution of 1 in 10. Anti-Fas blocking monoclonal antibody (Apotech, UK) was used at 10 µmol/L.

Cells
Mononuclear blood cells were obtained from normal bone marrow from individuals who donating cells for allogeneic transplantation. Written informed consent was obtained in all cases. The experiments investigated the effect of GM-CSF alone and in combination with G-CSF. The latter was used in all experiments because no colonies form without the presence of cytokines. Thus, G-CSF-stimulated colony-forming units of granulocytes–macrophages (CFU-GM) served as a baseline against which to compare the effect of the other cytokines. The CFU-GMs from normal bone marrow were grown in the presence of different growth factor combinations and the frequency of apoptotic cells was determined after seven days of incubation.

CFU-GM assay
Human bone marrow mononuclear cells were separated by centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway) and then depleted of adherent cells by incubation for two hours in plastic tissue culture flasks (Costar, Essex, UK). Non-adherent cells were plated (35 mm Petri dishes) at a concentration of $1x10^5$/mL in methylcellulose supplemented with recombinant human

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Table 1. Cytokine combinations used in the experiments

Cytokines	G-CSF 100 ng/mL	GM-CSF10 ng/mL
G-CSF (control)	+	
GM-CSF alone		+
G-CSF and GM-CSF	+	+

Table 2. Expression of Fas and Bcl-2 in $CD34^+$ cells.

GM-CSF concentration	Fas expression	Bcl-2 expression	No. samples
zero	–	–	*n*=6
10 ng/mL	+++	–	*n*=6
100 ng/mL		++	*n*=6

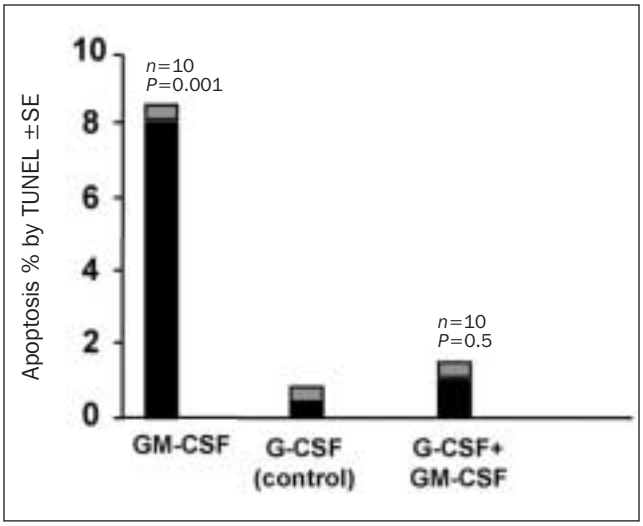

Fig. 1. Frequencies of apoptotic $CD34^+$ cells in CFU-GM, grown in the presence of GM-CSF alone, G-CSF alone and G-CSF+GM-CSF.

cytokines (Table 1). The Petri dishes were incubated at 37°C in humidified 5% CO_2 in air for seven days. Colonies of 50 or more cells were scored under an inverted microscope.

Separation of CD34+ cells

The CFU-GMs grown in methylcellulose for seven days were pooled in 3 µL MiniMacs (MM) buffer (PBS, Gibco) supplemented with 5 nmol/L EDTA and 0.5% bovine serum albumin (BSA; Gibco) and mixed vigorously to dissolve the methyllulose and disperse the cells. The cells were pelleted at 1800 rpm for 5 min. Separation of $CD34^+$ cells were achieved using immunomagnetic beads (MiniMacs; Miltenyi Biotec, Camberley, UK). Briefly, the cell pellet was resuspended in 30 µL MM buffer to which was added 10 µL reagent A1 and 10 µL reagent A2. After 15 min at 4°C, the cells were washed with 5 mL cold MM buffer and resuspended in 40 µL MM buffer. Reagent B (10 µL) was added, incubated at 4°C for a further 15 min and the cells were washed in 5 mL MM buffer and resuspended in 1 mL MM buffer. The cells were passed down a pre-flushed column in a magnetic field, which was then washed with 500 µL cold MM buffer (x4). After removal from the magnet, $CD34^+$ cells were eluted from the column with 1 mL MM buffer.

Terminal deoxynucleotidyl transferase dUTP nick end labelling

The $CD34^+$ cells suspended in MM buffer were mixed with an equal volume of 8% paraformaldehyde for 10 min. The cells were pelleted at 1800 rpm for 5 min then resuspended in Dulbecco's MEM (Gibco) at a concentration of $2x10^5$/mL, and 100 mL volumes were cytocentrifuged (Shandon Cytospin 2, Shandon, Pittsburgh, PA, USA) on to cleaned microscope slides at 450 rpm for 10 min. Slides were air dried overnight, rehydrated in TBS for 15 min at room temperature (RT) and dried.

For terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), the cells were covered by a 5-mL droplet of protein K diluted 1 in 100 in 10 mmol/L Tris (pH 8.0), incubated for 5 min at RT then dipped (x3) into TBS and then dried. The specimen was covered with 100 µL supplied equilibration buffer and incubated for 30 min at RT. Excess buffer was poured off and freshly prepared TdT labelling mixture (3 µL TdT enzyme in 57 mL TdT labelling reaction mix [Frag EL; Calbiochem, Nottingham, UK]) was layered on the cells. The slide was incubated at 37°C in humidified chamber for 1.5 h then washed (x3) in TBS at room temperature. A coverslip was applied over mounting medium (Frag EL) and sealed with nail varnish to prevent evaporation. At least 500 cells from randomly selected fields were scored by fluorescence microscopy (494 nm).

Direct immunofluorescence

The $CD34^+$ cells were examined by direct immunofluorescence to determine the expression of Fas and Bcl-2 proteins. For Fas expression 10^5 cells were cytocentrifuged onto slides and anti-Fas FITC-conjugated monoclonal antibody (PharMingen) was incubated with the fixed cells at a 1 in 10 dilution at 37°C for 1 h. For Bcl-2 expression, cells were fixed with 2% paraformaldhyde for 30 min at 4°C and then permeabilised with 75% ethanol prior to staining with anti-Bcl-2 FITC-conjugated monoclonal antibody (DakoCytomation) for 30 min at 4°C. Slides were washed twice with PBS for 10 min, mounted in glycerol and examined under an Olympus fluorescence microscope. Matched isotype antibodies were used as negative controls.

Statistical analysis

Statistical analysis was carried out using a Microsoft Excel spreadsheet and StatView SE+graphics software. Probability of a significant difference between groups was determined by Mann-Whitney U test and Wilcoxon signed rank test. Graphs were plotted using Cricket graph graphics package.

Results

Influence of cytokines

Figure 1 shows the effect of GM-CSF on the rate of apoptosis in $CD34^+$ cells and the effect obtained when G-CSF was added to GM-CSF, producing a level seen in CFU-GM stimulated by G-CSF alone (P=0.6).

Effect of cytokine concentration

Figure 2 shows that GM-CSF (100 ng/mL) alone or in combination could reduce the percentage of apoptotic cells

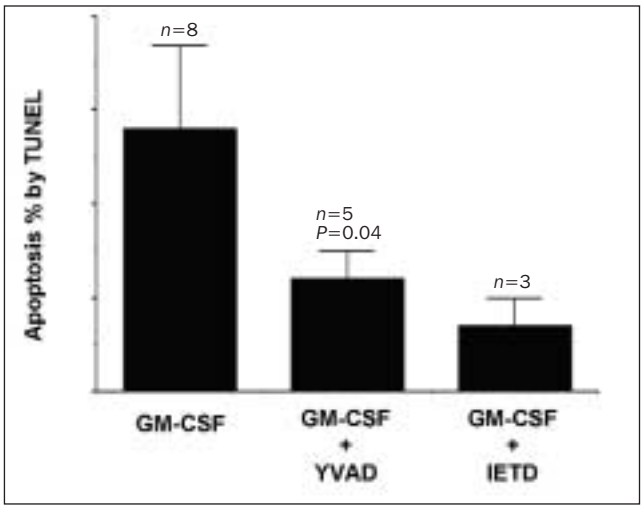

Fig. 3. Frequency of apoptotic CD34⁺ cells in CFU-GM, grown in the presence of GM-CSF alone and YVAD+GM-CSF or IETD+GM-CSF.

to the level seen with G-CSF alone. This was also the case when a lower concentration of GM-CSF (10 ng/mL) was used alone or in combination with G-CSF. This suggests that G-CSF and GM-CSF have different specific abilities to protect cells from apoptosis.

Effect of caspase inhibitors

To determine whether or not the observed effects of cytokine exposure on apoptosis of CD34⁺ cells could be related to activation of caspase cascade, YVAD (a caspase-1, ICE-like protease inhibitor; Bachem, UK)[5–9] or IETD (a caspase-8 specific inhibitor; IETD-cho, BD, UK)[10] was added to GM-CSF and the subsequent effect of G-CSF in blocking the apoptotic pathways was assessed. Results showed that YVAD (10 µmol/L) or IETD (10 µmol/L) had the same effect as G-CSF (Fig. 3) in reducing the frequency of apoptosis in CD34⁺ cells.

Caspase-8, which may be activated via caspase-1, has been described as one of the keys to apoptosis and has been demonstrated to activate terminal caspases.[11] The results present here indicate that caspase-1- and caspase-8-mediated apoptosis pathway is activated in CD34⁺ cell apoptosis during colony formation *in vitro*, and that its level of activation can be modulated by cytokines. Caspase-1 and caspase-8 play an important role in inducing apoptosis, as both are members of the interleukin-1β converting enzyme (ICE) family, which is involved in the induction of apoptosis.

Effects of cytokines on actinomycin D-mediated apoptosis

The CD34⁺ cells were incubated with the apoptosis inducer actinomycin-D (50 µmol/L), with and without G-CSF and/or GM-CSF. The data in Figure 4 show that G-CSF and GM-CSF were equally effective in the test system used. This indicated that the concentration-dependent effectiveness of GM-CSF in protecting cells from apoptosis may be different in methylcellulose and liquid media, and that the mechanism of actinomycin D-mediated apoptosis differs from that regulating apoptosis during GM-CSF colony formation.

Effect of culture media on CD34⁺ cell apoptosis

The data showed that the density of the surrounding medium influenced the observed level of apoptosis in CD34⁺ cell (Fig. 5). The differences observed may reflect the

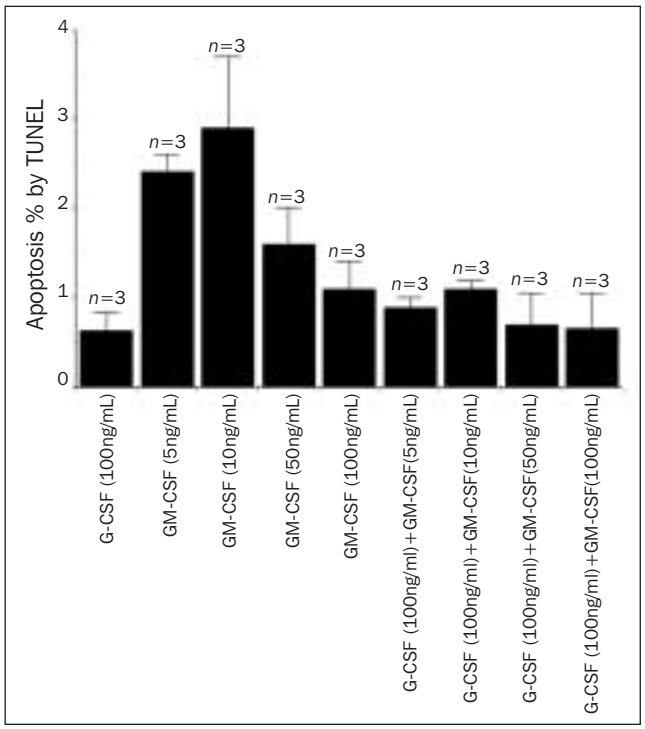

Fig. 2. Frequencies of apoptotic CD34⁺ cells in CFU-GM, grown in the presence of increasing concentrations of GM-CSF alone or with G-CSF.

different conditions for cell-cell interaction or the 'juxtacrine' effect[12–14] of secreted soluble factors.[15–17] The same serum was used for liquid and methylcellulose cultures.

Effect of cell crowding

A standard number ($2.5x10^5$) of CD34⁺ cells were incubated in 96-well plates, 48-well plates and 35 mm diameter Petri dishes in the presence of GM-CSF for 24 hours before being transferred to methylcellulose cultures for CFU-GM assay and analysis by TUNEL. The results are presented in Figure 6; however, the experiment did not discriminate between the effect of cell-cell contact and the effect of soluble factors.

Effect on Fas and Bcl-2 expression

The CD34⁺ cells were analysed for the expression of Fas and Bcl-2 by immunofluorescence (Table 2). Fas expression in CD34⁺ cells following exposure to 10 ng/mL GM-CSF is shown in Figure 7. The effect of GM-CSF appeared to be biphasic, whereby Fas was up-regulated at 10 ng/mL GM-CSF but was undetectable at 100 ng/mL.

Effects of anti-Fas blocking

Results showed that anti-Fas blocking monoclonal antibody inhibited apoptosis in CD34⁺ cells (Fig. 8, $P=0.05$).

Discussion

It is generally accepted that haemopoietic growth factors such as G-CSF and GM-CSF maintain not only cell proliferation and differentiation but also cell survival.[18] Dexter *et al.*[18] demonstrated that GM-dependent and G-CSF-dependent cell lines undergo rapid apoptosis after withdrawal of relevant colony-stimulating factors. Similarly,

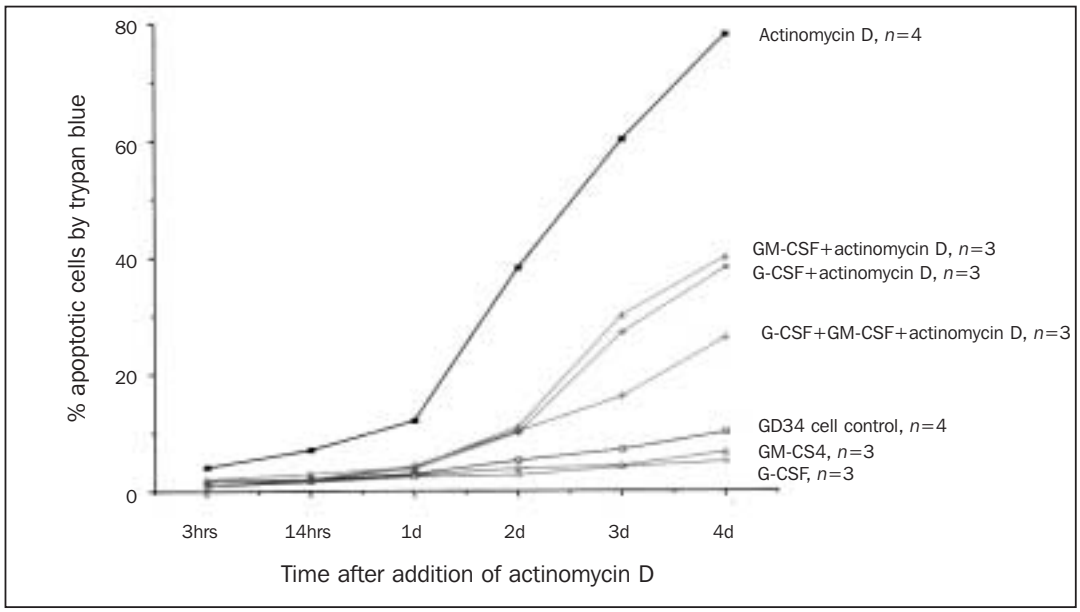

Fig. 4. Survival of CD34⁺ cells in liquid cultures, grown in the presence of 100 ng/mL G-CSF and/or 10 ng/mL GM-CSF as measured by trypan blue dye exclusion.

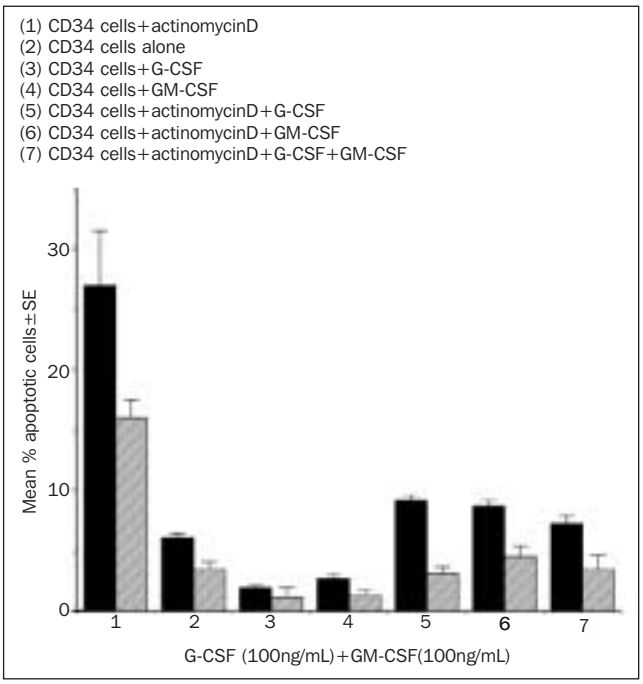

Fig. 5. Percentage of apoptotic CD34⁺ cells grown in the presence of 100 ng/mL G-CSF and/or 10 ng/mL GM-CSF after four days in methylcellulose medium (solid columns) and in liquid culture (striped columns).

Urashima *et al*.[19] showed that deprivation of GM-CSF in a TF-1 cell line (a GM-CSF-dependent cell line) induced apoptosis. Dexter's group also demonstrated that IL-3 deprivation over two days of incubation resulted in cell death in the FDCP-1 haemopoietic precursor line (IL-3-dependent cells) characterised by morphological features such as DNA fragmentation and plasma membrane blebs.

Park[20] reviewed apoptosis as a mechanism for limiting precursor cell survival and showed that while some cytokines prevent apoptosis in haemopoietic cells (e.g.. IL-3, G-CSF), others may induce apoptosis (e.g., TNFα). Silva and colleagues[21] demonstrated that when the murine Epo-dependent cell line HCD-57 is cultured in the absence of Epo, the cells undergo cell death, and Bcl-2 and Bcl-xL expression is down-regulated. Also, withdrawal of IL-3 from murine 32D myeloid cells (an IL-3-dependent cell line) resulted in rapid apoptosis that was dependent on the expression of wild-type p53.[22] Bradbury *et al*.[23] and Klampfer *et al*.[24] showed that primary CD34⁺ cells are more resistant to apoptosis cells following exposure to GM-CSF because of their ability to up-regulate Bcl-2.

Results from the present study suggest that GM-CSF might be a pro-apoptotic agent or a much less effective anti-apoptotic agent than G-CSF. In addition, results obtained from clonogenic assays showed that an increase in the percentage of apoptotic cells in the presence of GM-CSF at 5 and 10 ng/mL, and this effect was reduced by adding G-CSF to GM-CSF or by increasing the concentration of GM-CSF. The results using YVAD and IETD showed that CD34⁺ cell death in colonies occurs via a pathway involving caspase-1 and caspase-8 activation.

Maianski *et al*.[25] demonstrated that G-CSF delayed polymorphonuclear neutrophil apoptosis, prevented translocation of Bax to mitochondria and the activation of caspase-3. Similarly, Mende *et al*.[26] showed that treatment of bone marrow cells with G-CSF reduced Fas-mediated caspase-8 and caspase-3 activation significantly. Recent evidence provided by Ottonello *et al*.[27] showed that GM-CSF inhibits spontaneous neutrophil apoptosis and Bax up-regulation.

Goyal *et al*.[28] demonstrated that GM-CSF suppresses DNA fragmentation and caspase-3 and caspase-9 activation, and Liu and colleagues[29–30] showed that GM-CSF withdrawal leads to caspase-3 activation and DNA degradation. In contrast, at least one study has linked the presence of GM-CSF to caspase activation. Okuma *et al*.[31] showed that GM-CSF induced apoptosis in a human U937 cell line; however, apoptosis was not inhibited by YVAD-cmk, DEVD-cho or ZVAD-fmk under the same conditions in which these inhibitors suppress TNF-induced apoptosis.

In contrast to the results obtained in the clonogenic assay system, GM-CSF and G-CSF were similarly effective in preventing apoptosis in liquid cultures of CD34⁺ cells,

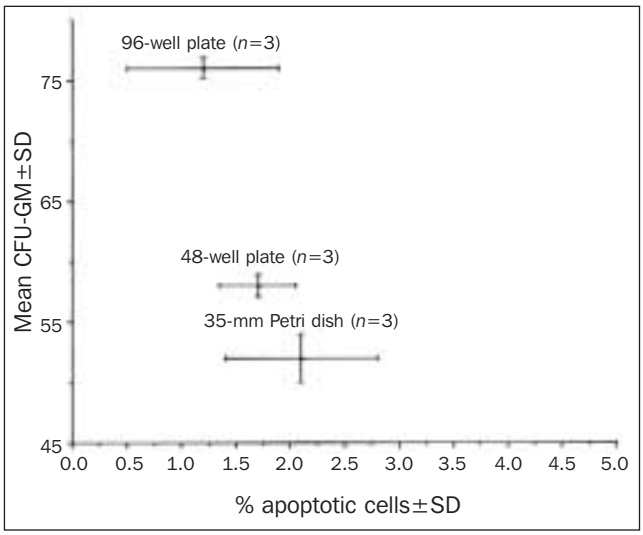

Fig. 6. Relationship between apoptosis and number of CFU-GM under different cell crowding conditions at 10 ng/mL GM-CSF.

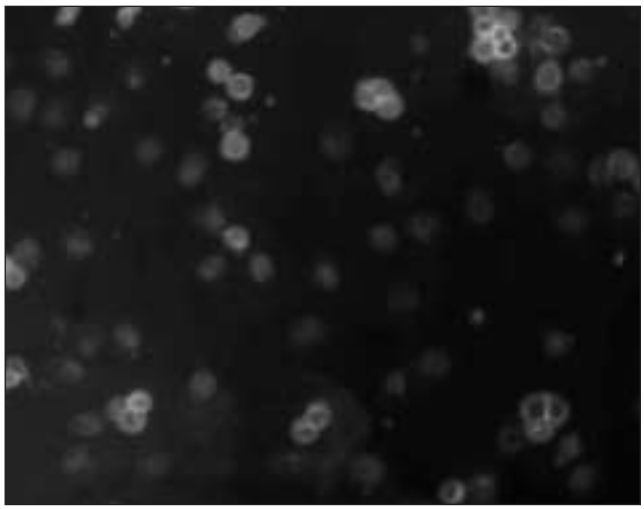

Fig. 7. CD34⁺ cells incubated in the presence of GM-CSF at 10 ng/mL for two days. Fas expression stained with FITC-conjugated anti-Fas monoclonal antibody by direct immunofluorescence.

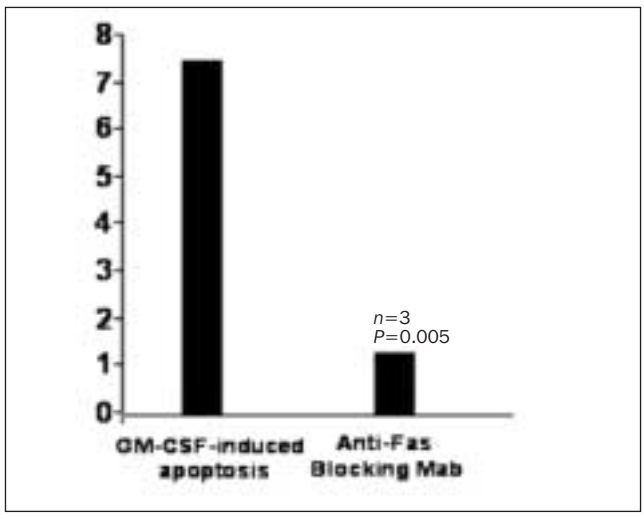

Fig. 8. CD34⁺ cells incubated in the presence of GM-CSF at 10 ng/mL for two days in the presence of the blocking anti-Fas monoclonal antibody.

irrespective of treatment with actinomycin-D. Variation in results between liquid and methylcellulose cultures might be explained by the fact the actinomycin-D can kill cells by either cytochrome C release or via the Fas/FasL pathway.

Results obtained from the liquid cultures indicated that CD34⁺ cells survived better at high density in liquid medium, decreasing apoptotic rate significantly. In contrast, CD34⁺ cells on methylcellulose were dispersed, which might have been due to secretion of soluble factors released in juxtacrine and/or autocrine fashion. Further investigation using enzyme-linked immunosorbent assay (ELISA) as a quantitative assay and Western blotting as a qualitative assay is warranted.

Ishizaki *et al*.[35] demonstrated that lens epithelial cells can survive for weeks at high density in the absence of other cell types or of serum or protein but require signals from other epithelial cells to survive. Medium from high-density cultures, fetal calf serum (FCS) or a combination of growth factors all prolonged chondrocytes survival in low-density culture. When grown in agarose gel, their survival depended on cell-density. This suggests that lens epithelial cells support each other's survival by secreting survival factors. Therefore, CD34⁺ cell density, may also contribute to the regulation of cell death.

Signalling mechanisms influenced by (or mediated through) cell–cell contact or the cellular environment may play an important role in CD34⁺ cell sensitivity to apoptosis. In the present study, an effect of cell crowding was also seen when cells were grown in methylcellulose cultures (35 mm Petri dishes) and in liquid culture (96-well plates). Cell crowding in 96-well plates resulted in fewer apoptotic cells and better CFU-GM survival than was apparent in less-crowded cell culture conditions. It is possible that CD34+ cell crowding initiates survival signals and/or blocks apoptotic signals, perhaps through the activation of cytoplasmic molecules such as Jak2 and PI3. These pathways lead to survival and proliferation.[36]

Impaired cell-matrix contact, leading to apoptosis has been termed anoikis[37] and was found to be involved in a wide range of tissue homeostatic, developmental and oncogenic processes. Most epithelial cells are normally attached to a basement membrane and are sensitive to anoikis. Conversely, carcinoma cells tend to be resistant to anoikis and this probably plays a role in tumour metastasis. However, little is known about the signalling events implicated in the induction and regulation of anoikis in haemopoietic cells.

The level of Fas expression was highest in CD34⁺ cells exposed to 10 ng/mL GM-CSF, whereas Bcl-2 expression was seen in CD34⁺ cells exposed to 100 ng/mL GM-CSF. The extent to which apoptosis was induced by GM-CSF correlated strongly with the number of cells that expressed Fas (data not shown). Addition of blocking anti-Fas monoclonal antibody significantly reduced GM-CSF-induced apoptosis. These results indicate a contribution of the Fas system to GM-CSF-mediated apoptosis.

In conclusion, the results presented here suggest that low concentrations of GM-CSF induce a level of Fas expression that may be sufficient to trigger caspase-8-dependent apoptosis. However, the mechanisms by which GM-CSF induces CD34⁺ cells apoptosis are complex and appear to be influenced by factors including cytokine concentration, cell culture conditions and crowding, and cell interactions.

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