INTRODUCTION

Regulation of the level of apoptosis in hemopoiesis could potentially provide a mechanism for regulating stem and progenitor cell population size. Thus, if there is substantial overproduction of stem or progenitor cells and compensatory loss by...
apoptosis during normal hemopoiesis, it would be possible to increase mature cell production in response to demand (e.g., anemia, neutropenia), by reducing the level of apoptotic cell loss. It would be also be possible for dysregulation of apoptosis to lead to leukemia.

One of the best characterized apoptotic signalling cascades follows the engagement of FasR/CD95 with its ligand FasL/CD95L [1-5]. FasR (CD95) is a 48 kDa cysteine rich type I cell membrane protein, a member of the TNF/NGF receptor gene family which also includes TNFR, DR3, DR4, DR5, DR6 and APRIL.

FasR is broadly expressed on a variety of cells including liver, lung, intestine, heart, kidney, ovary, various lymphomas, and leukemic cells [6-8]. In contrast, the expression of Fas ligand (FasL) † appears to be more restricted to natural killer (NK) cells, cytotoxic T lymphocytes (CTL) [9], and cells in immunoprivileged sites such as the eye [10].

FasR, TNFR1 and other family members bear in their cytoplasmic domain motifs known as death domains (DD), which associate with each other to form trimers following ligation with their corresponding ligands. This association seems to be the first step in signal transduction. The aggregated DD of FasR recruits the DD on the adaptor protein Fas-associated death domain (FADD). In the TNFR pathway, an intermediate molecule “TNFR-associated death domain” (TRADD) interacts with FADD. Together, Fas and FADD form a death-inducing signalling complex (DISC) with FADD like ICE (FLICE)/caspase-8. The N-terminal end of FLICE consists of two death effector domains (DED) [11-12]. Activation of DED leads to the activation of terminal caspases-3, -6 and -7, and subsequently to degradation of lamin, actin, and poly ADP-ribose polymerase (PARP).

Most publications on the subject show that the anti-Fas Mab CH-11 induces apoptosis by activating the Fas pathway [13-14]. Consequently, CH-11 might be expected to reduce proliferation by colony forming unit-granulocyte and macrophage (CFU-GM). However, a study by Barcena and colleagues reported that the anti-Fas CH-11 Mab induced a dose-dependent proliferative response by CD34+CD38- cells and significantly increased the number of CFU-GM colonies generated by hemopoietic stem cell (HSC) in liquid culture [15]. This observation might suggest that in some circumstances CH-11 blocks Fas. Similarly, sFasL was found to enhance the generation of cells of the erythroid and granulocytic lineages and also to increase the viability of CD34+CD38- cells [16]. Therefore, their results indicate that Fas/FasL system may regulate human hemopoietic progenitor cells in a way that is closely similar to the mechanism suggested by Alenzi et al. [17] for murine progenitor cells.

Other evidence also supports the involvement of Fas and FasL in regulating hemopoiesis. Peschle’s group [18] has demonstrated the possible involvement of Fas and FasL in the regulation of erythropoiesis by immunohistochemistry of normal bone marrow samples, which revealed that immature erythroblasts undergo apoptosis in vivo. These findings suggest the existence of a negative regulatory feedback between mature and immature erythroid cells. Accordingly, the interaction of Fas and FasL might represent an apoptotic control mechanism for erythropoiesis, contributing to the regulation of red blood cell homeostasis. Traver et al. [19] proposed that granulopoiesis could be regulated similarly by a negative feedback mechanism through interactions between Fas-expressing clonogenic cells and neutrophils that express FasL.

Caspase-8 inhibitor (IETD) was added to the CFU-GM cultures. It is involved in the induction of apoptosis and has been considered to be correlated with apoptosis because of the most downstream enzyme in their apoptosis-inducing pathway. IETD was used to inhibit apoptosis
during CFU-GM colony formation and so blocks caspase activation.

The work described in this study was directed toward determining whether apoptosis mediated by Fas and FasL influences progenitor cell kinetics as reflected in the AUC assay, i.e., proliferation in humans. There is evidence in the literature that Fas and FasL influence human hematopoietic progenitor cell numbers in different in vitro systems, but their effects on human progenitor cell proliferation kinetics have not been investigated before.

The purpose of this study was to address the question of whether the Fas/FasL system is likely to be involved in the regulation of myeloid progenitor cell population size in human model. First, a role for apoptosis was investigated by exposing normal bone marrow (NBM) CFU-GM to IETD. Second, the plating efficiency of CFU-GM and its proliferative capacity was determined after being exposed to anti-Fas Mab CH-11. Thirdly, the expression of Fas and FasL in human CD34+ cells and CFU-GM respectively was investigated. For this, CD34+ cells from NBM samples were grown in methylcellulose cultures.

**MATERIALS AND METHODS**

**Sources of cells**

NBM samples were obtained from individuals donating cells for allogeneic transplantation. Written informed consent and Research Ethics Committee approval were obtained in all cases. Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard, Oslo, Norway). After washing with HBSS (GibcoBRL, Carlsbad, California, United States), cells were resuspended in HBSS. To calculate the concentration of nucleated cells, the sample was diluted with 3 percent acetic acid to lyse red blood cells, and an aliquot placed in a hemocytometer counting chamber. The cell concentration was adjusted to 10 x 10^6/5 ml with 15 percent FCS in minimal essential medium (MEM) (GibcoBRL). The cell suspension was placed in a 25 cm^2 plastic flask for two-hour incubation at 37°C in humidified 5 percent CO_2 in air to remove adherent monocytes and macrophages. Non adherent MNCs were harvested for culture.

**Sources of recombinant cytokines and growth factors**

Recombinant human granulocyte-colony stimulating factor (G-CSF; 100 ng/ml), granulocyte-macrophage-colony stimulating factor (GM-CSF; 10 ng/ml), interleukin-3 (IL-3; 25 ng/ml), and stem cell factor (SCF; 100 ng/ml). All recombinant cytokines were obtained from First Link (West Midlands, United Kingdom). The concentration of each cytokine used was optimized in the laboratory.

**Reagents**

IETD (Bachem, United Kingdom) was used at 10 M; anti-Fas Mab CH-11 was purchased from Upstate Biotechnology (Lake Placid, New York); anti-FasL Mab (65321A) was purchased from PharMingen (San Diego, California, United States), sFasL was purchased from Alexis (San Diego, California, United States); Blocking anti-Fas Mab ZB4 from MBL was used at 1 ng/ml (Nagoya, Japan); and irrelevant IgG1 control Mab (PharMingen) served as isotype-matched control.

**Clonogenic CFU-GM assay**

Non-adherent MNCs were mixed with 3 ml of methylcellulose (MC) (1 x 10^5 cells/ml) (StemCell Technologies, Grenoble, France) containing 10 percent FCS and supplemented with recombinant human cytokines (IL-3 [5 ng/ml] and SCF [20 ng/ml]). After being mixed well, 1 ml aliquots were plated out into three 35 cm^2 diameter petri dishes. The dishes were then incubated at 37°C in humidified 5 percent CO_2 in air for seven days. Colonies consisting of 50 cells or more were scored under an inverted microscope, and
results are expressed as the number of CFU-GM per 10⁵ cells.

**Purification of human CD34⁺ cells**

CD34⁺ cells were separated using MiniMACS magnetic immuno-affinity columns as directed by the manufacturer (Milteny Biotec, Auburn, California). Cells from pooled day 7 CFU-GM were incubated for 15 min at 4°C with 100 µl reagent A1 (blocking FcR), 100 µl of reagent A2 (hapten-conjugated anti CD34⁺ Mab), and 300 µl of MiniMacs buffer (MM) containing calcium and magnesium-free PBS (GibcoBRL), 0.5 percent human serum albumin (ImmunoAG), and 5 mM EDTA (GibcoBRL). Following a single wash in 10 ml MM, cells were resuspended with 100 µl reagent B (anti-hapten Mab conjugated to beads) and 400 µl of MM buffer and incubated for 15 minutes at 4°C. Cells then were washed again in 10 ml MM and gently but thoroughly resuspended in 1 ml of MM buffer. The labeled cells were loaded onto the affinity column held in a magnet and CD34⁺ cells washed through with 4 x 0.5 ml of MM buffer. After removal from the magnet, the CD34⁺ cells were vigorously expelled from the column with 1 ml MM buffer. The purity is about 85 percent to 98 percent, confirmed by flow cytometry.

**CFU-GM replating assay**

After seven days of incubation, 120 colonies consisting of 50 cells or more were plucked individually from methycellulose using a sterile eppendorf pipette and thoroughly dispersed in separate wells of a 96-well plate, each containing 100 µl of MC plus FCS and supplemented with IL-3 (5 ng/ml), G-CSF (100 ng/ml), GM-CSF (1 ng/ml), and SCF (20 ng/ml). The surrounding wells were filled with sterile water to prevent evaporation during incubation. Plates were incubated for a further seven days at 37°C in humidified 5 percent CO₂ in air. Then each well was scored for secondary colonies of more than 50 cells.

The results were plotted as the cumulative distribution of the proportion of primary CFU-GM producing more than (n) secondary colonies. Both axes are expressed on a logarithmic scale. This step allows good data fit, so the area under the curve (AUC) can be measured using the Trapezium Rule using Microsoft Excel version 5.0 on a Macintosh computer. The AUC is measured because the distribution of secondary colonies is highly skewed rather than being normally distributed. Therefore, the median and not the mean is the correct value to use. However, for cases where more than 50 percent of the replated colonies do not yield secondary colonies, the median would be zero and uninformative.

The AUC is calculated to provide an overall measure of progenitor cell self-renewal capacity and is particularly useful in situations where this capacity is suppressed.

**Detection of Fas by reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from a constant number of CD34⁺ cells-derived CFU-GM at day seven of incubation using a Qiagen gel purification kit, according to the manufacturer’s instructions. First-strand cDNA synthesis and PCR were performed. For the amplification, PCR primers were used (see below).

**Fas R:** 5′-CAA GTC CAA CTC AAG GTC CATGCC-3′ (bases no. 517-540).

**Fas F:** 5′-CAG AGA GAG CTC AGA TAC GTT GAC-3′ (bases no. 839-862).

Thirty-five cycles of PCR, with denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, were performed on a programmed-temperature system (Hybaid OmniGene; Midwest Scientific, Missouri, United States).

After PCR amplification, 10 µl of the PCR products were mixed with 2 µl of 6X
DNA loading buffer (0.25 percent bromophenol blue, 40 percent w/v sucrose in water) and electrophoresed on a 2 percent agarose gel containing 0.2 µg/ml ethidium bromide in 0.5X TBE buffer. A DNA ladder was also run in parallel. The amplified gel was visualized and photographed under UV light.

**Detection of FasL by direct immunofluorescence**

Direct immunofluorescence was used to confirm the presence of FasL. 10^5 cells were deposited on slides using a cytocentrifuge and stained with an anti-FasL FITC conjugate (PharMingen). The cells were incubated with the antibody at 1:100 dilution at RT for 1 hour.

Slides were washed three times with PBS for 5 minutes, mounted with glycerol mountant and checked under an Olympus fluorescence microscope. Matched isotype antibody control was used at 1:100 at RT for 1 hour.

**Statistical analysis**

Statistical analysis was carried out using Microsoft Excel spreadsheet and the StatView SE+ graphics software. The probability of a significant difference between groups was determined by Wilcoxon signed rank test.

Graphs were plotted using the Cricket Graph graphics package. The AUC was calculated using a Microsoft Excel spreadsheet. All software was run on a Macintosh computer.

**RESULTS**

**Effect of IETD on CFU-GM plating efficiency in methylcellulose cultures**

To determine whether caspases could play a role in the terminal myeloid differentiation, we used a human bone marrow-derived CFU-GMs grown in the presence of IETD for 7 days. As may be seen from Figure 1, IETD significantly increased the AUC. However, the 1.6-fold increase was less marked than the 2.5-fold increase in WT mouse cultures [17]. This result indicates that apoptosis may play a role in regulating hematopoietic progenitor cell kinetics in humans as it does in mice. This observation suggested also that caspase activation was required for the myeloid maturation.

**Expression of Fas on CD34^+ cells-derived day 7 CFU-GM**

We determined whether Fas is expressed in CD34^+ cells-derived day 7 CFU-GM by RT-PCR. Fas was detected in almost all obtained CD34^+ cells (Figure 2).

**Expression of FasL on day 14 CFU-GM**

Experiments were performed first to demonstrate that FasL was present in day 14 CFU-GM cells. To investigate the expression of FasL protein in day 14 CFU-GM, they were deposited on slides using a cytocentrifuge and stained with an anti-FasL FITC conjugate (PharMingen). The cells were incubated with the antibody.
Approximately 90 percent of day 14 CFU-GM expressed FasL (Figure 3). To exclude any interference, normal FITC-IgG was set as a control (data not shown).

**Demonstration of functional Fas**

Barcena et al. and others [15-16] reported that the anti-Fas Mab CH-11 significantly increases the number of CFU-GM colonies generated from HSC in liquid culture in a dose-dependent manner over a range of 1 to 10 µg/ml. Therefore, this Mab CH-11 was used to investigate whether the effects of IETD (Figure 1) could be related to the Fas/FasL pathway.

To assess the effect of Mab CH-11 on cell death, 10^6 Jurkat T cells were incubated with anti-Fas Mab CH-11 at 1 µg/ml and 0.1 µg/ml. The percentage of apoptotic cells was calculated using the trypan blue dye-exclusion test (> 40 percent in 3 days). As a positive control, the effects of 1 µg/ml anti-Fas CH-11 Mab on Jurkat cells were examined. This result identified CH-11 as an effective pro-apoptotic reagent in the Jurkat cell line model (data not shown).

When CH-11 was added to clonogenic cultures in methylcellulose, there was a trend effect on the CFU-GM plating efficiency at 10 µg/ml (Figure 4). Therefore, CH-11 does not appear to have any significant cytotoxic or stimulatory effects on primary colony formation when the cells are plated into methylcellulose straight away. Additionally, the results in Table 1 shows that there are differences with regard to the composition, i.e., cell number of day seven and day 14 colonies, between treated and non-treated cells in the presence of ascending concentrations of anti-Fas Mab.

The influence of CH-11 on proliferation (the AUC) by CFU-GM grown in its presence was then investigated. At 10 g/ml, CH-11 significantly reduced the replating ability (AUC) by 30 percent to 0.71 ± 0.1 (m ± se) of the control values. Neither 1 µg/ml nor 0.1 µg/ml had any significant effect. The lower dose of anti-Fas Mab 0.01 g/ml, significantly increased the replating ability (AUC) (p = .02) (Figure 5). This result suggests that CH-11 may have a biphasic effect on progenitor cell

**Figure 2.** Expression of Fas into CD34+-cells-derived CFU-GM was determined by RT-PCR. Ten day 7 CFU-GM colonies were collected and tested by PCR. Ten colonies were PCR-positive.

**Figure 3.** Immunofluorescence staining showing the FasL expression on day 14 CFU-GM, when stained with anti-FasL FITC Mab, compared to cells stained with isotype control (not shown). Data are a representative of four experiments.
proliferation. The stimulatory effect was, however, observed only at very low antibody concentration. It is noteworthy that no stimulation of primary CFU-GM plating efficiency was seen at this concentration (see Figure 4). The inhibitory effect seen at 10 µg/ml is consistent with a pro-apoptotic effect of CH-11 and a role for Fas in regulating human hemopoiesis.

Our results suggest that CH-11 is functioning to block FasL interaction with Fas. This suggestion has been confirmed using commercial blocking anti-Fas ZB4 Mab to Fas (Figure 6). The biphasic response to CH-11 is interesting, therefore it was of importance to validate this by incubating CD-34 cells with CH-11 at the various concentrations in liquid culture and measuring apoptosis (Figure 7). sFasL was utilized as a positive control (Figure 8).

**DISCUSSION**

The regulation of hemopoietic stem and progenitor cell numbers involves the balance between proliferation, differentiation, and apoptosis [20]. Thus, two main mechanisms may account for an increase in hemopoietic stem/progenitor cell numbers. The first, a reduction in progenitor cell loss by apoptosis, supposes that substantial numbers of them are lost by apoptosis in steady-state hemopoiesis [21]. The second is an increase in progenitor cell numbers.

| Incubation period | CH-11 Mab 10 mg/ml | CH-11 Mab 1 mg/ml | CH-11 Mab 0.1 mg/ml | CH-11 Mab 0.01 mg/ml | Control |
|-------------------|------------------|------------------|------------------|-------------------|--------|
| Number of CFU-GM at 7 days (M ± SE) | 281 ± 25 | 266 ± 59 | 228 ± 69 | 259 ± 10 | 264 ± 23 |
| Number of CFU-GM at 14 days (M ± SE) | 655 ± 15 | 910 ± 24 | 1011 ± 09 | 864 ± 7 | 912 ± 2 |
| Frequency and Significance | N = 6 | N = 6 | N = 5 | N = 6 | N = 6 |
|                             | P = .04 | P = .02 | P = .001 | P = .05 | P = .04 |
proliferation at the expense of differentiation. Until recently, studies on the involvement of Fas and FasL in hemopoiesis have focused on their proapoptotic functions [22-25]. However, some reports indicated that activation of downstream caspases can exert regulatory effects in the absence of cell death [26]. This led us to investigate the influence of Fas and FasL on the regulation of myelopoiesis. We evaluated the influence of the Fas and FasL apoptotic pathway on myeloid progenitors in primary and secondary CFU-GM obtained from normal human bone marrow. We investigated the hypothesis that Fas and FasL and downstream caspase activity play a role in regulating myeloid progenitors proliferation and differentiation.

IETD is a specific inhibitor of caspase-8. The finding that IETD significantly increased the AUC to the controls, but its effect on human cells was less marked than its effect on murine cells [17]. It is of relevance and interest that caspase-1 has been shown to participate in cleaving pRb [27-29], which is involved in regulating progenitor cell proliferation, differentiation, and hemopoiesis [30-31]. More interestingly, caspase-3 has been shown to be associated with altered expression of cyclin-dependent kinase inhibitors: p16, p21, and p27 [32-33], which also influence progenitor cell proliferation and differentiation [34].

Fas and FasL are a very important cellular pathways responsible for initiation of apoptosis. Fas is 45 kDa membrane glycoprotein and is widely distributed; in contrast, FasL is 40 kDa and is relatively restricted to some tissues. We studied the
expression of both Fas and FasL in CD34+ cells and in day 14 CFU-GM using RT-PCR and direct immunofluorescence respectively.

To investigate whether the Fas Mab CH-11 modulates proliferation of CFU-GM cells in NBM, we have used a commercially available Fas Mab CH-11. This reagent has been used by two other groups [15-16] who showed that it stimulated human hemopoietic progenitor cell proliferation although it is generally accepted that Fas ligation often leads to massive apoptosis in Fas-expressing cells [13-14]. Ligation of Fas with CH-11 Mab markedly reduced the AUC, indicating that Fas expression was associated with functional inhibition of CFU-GM. Therefore, these observations suggest that the Fas/FasL system is responsible for at least part of the inhibition of myelopoiesis in human CFU-GM.

Primary CFU-GM(s) were grown in the presence of CH-11, then replated for secondary colonies. CH-11 had no affect on primary CFU-GM. In the replating assay, CH-11 had a biphasic effect. Increased proliferation of progenitor cells was observed at the lowest concentration used 0.01 µg/ml anti-Fas CH-11 Mab in methylcellulose culture. It is possible, therefore, that anti-Fas Mab suppresses proliferation at higher

Figure 7. One million CD34+ cells were incubated with descending concentrations of human anti-Fas CH-11 Mab. The percentage of non-viable cells was scored using the trypan blue dye-exclusion test. The figure shows that CH-11 is a strong inducer of cell death.

Figure 8. One million CD34+ cells were incubated with sFasL at 100 ng/ml for 6 hours, 1 day, and 2 days. The percentage of non-viable cells was scored using the trypan blue dye-exclusion test. The results showed that sFasL induced cell death significantly to the level of the control.
concentrations, probably due to an ability to mimic apoptotic downstream signals. In contrast, at lower concentrations, it may block access of FasL to Fas and lead to a proliferative effect.

Also, since Fas is a multifunctional protein belonging to the TNF superfamily, it can trigger and activate two separate pathways. Fas binds to FADD death domains, leading to recruitment of caspase-8, which eventually leads to apoptosis. It should be noted, however, that 0.01 g/ml is much lower than the lowest concentration used by Barcena et al. when they demonstrated a proliferative effective of CH-11. Although the exact role of NFκB in apoptosis is not fully understood, two pieces of evidence demonstrated that NFκB activation can inhibit apoptosis in neuron cells [35] and non-neurons cells [36]. Chu et al. [37] demonstrated that TNF-receptor-associated protein 2 (TRAF2) interacts with the inhibitors of apoptosis proteins (IAP), which have been shown to increase the levels of NFκB. Other evidence is provided by Ravi et al [38] who demonstrated that NFκB was activated by Fas. The former evidence suggests that IAP could abolish apoptosis directly by inhibiting caspase activation, or alternatively via increased activation of NFκB.

The results reported in this study do not support the findings of Barcena et al. or Josefsen et al. because no convincing proliferative effect of CH-11 was found in any of the progenitor cell culture systems tested [15-16]. This may not be surprising in view of the fact that CH-11 was identified as an effective pro-apoptotic reagent in the Jurkat cell line model. However, CH-11 did not have any striking effects on progenitor cells that would be consistent with a pro-apoptotic effect either, with the exception of its negative influence on the replating ability of CFU-GM (Figure 5). Importantly, the 30 percent reduction in AUC shown in Figure 3 is consistent with the increase in AUC seen in the presence of IETD (Figure 1). Additionally, most of CFU-GM in our cultures at day 14 are FasL+ cells.

Our results are contradicted by Kim et al. [39] who demonstrated that CD34+ cells do not express Fas and that they are resistant to anti-Fas Mab and sFasL-induced apoptosis. Moreover, they showed that CD34+ cells express FLIP which protects from Fas-mediated apoptosis. Upon GM-CSF deprivation or Fas activation, caspase activity is amplified via either the caspase-9 pathway or the caspase-8 pathway, respectively, which leads eventually to apoptosis. Therefore, in this study, we have described a new function for caspases as regulators for myeloid proliferation.

Overall, our results suggest that the Fas/FasL pathway and caspase activation inhibit cell proliferation and promote differentiation. Therefore, our results support the previous proposals that the caspase activation may have nonapoptotic functions in the regulation of hemopoiesis [26].

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