The Cytokine, Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF), Can Deliver Bcl-XL as an Extracellular Fusion Protein to Protect Cells from Apoptosis and Retain Differentiation Induction

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Bcl-XL, a member of the Bcl-2 family, is able to suppress cell death induced by diverse stimuli in many cell types, including hematopoietic cells. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors. We fused GM-CSF to Bcl-XL and examined the capacity of this chimera to bind human cells through the GM-CSF receptor and prevent apoptosis. We found that the chimeric protein increased the proliferation of human monocytes in culture from 24 h until at least 72 h. In the presence of different apoptotic agents, GM-CSF-Bcl-XL protected cells from induced cell death and promoted proliferation, whereas GM-CSF alone was completely inhibited. In the presence of cytarabine, GM-CSF-Bcl-XL was able also to promote the differentiation of the CD34+ myeloid precursor whereas Lfn-Bcl-XL, lacking the GM-CSF domain-stimulated cell proliferation and not differentiation. We conclude that recombinant GM-CSF-Bcl-XL binds the GM-CSF receptor on human monocyte/macrophage cells and bone marrow progenitors inducing differentiation and allowing Bcl-XL entry into cells where it blocks cell death and allows amplified cell proliferation. This fully human fusion protein has potential to prevent monocytopenia and represents a new strategy for engineering anti-apoptotic therapeutics.

Bcl-2 family proteins are central regulators of programmed cell death. Their function is to integrate survival and death signals that are generated inside and outside cells and mediate the commitment step to cell death. After this point, an execution phase begins with release of cytochrome c from mitochondria and caspase activation. Downstream caspase activation triggers the morphological and biochemical changes associated with efficient cell catabolism (1).

Bcl-XL is a well characterized member of the Bcl-2 family protein that is able to suppress cell death induced by diverse stimuli in many cell types (2, 3). In healthy cells Bcl-XL is found in the cytosol and attached to the mitochondrial membrane where it inhibits Bax and Bak activity (4). In addition, Bcl-XL in the cytosol appears able to sequester BH3 domain-only molecules thus preventing the activation of Bax, Bak, and apoptosis (5–7). Furthermore, Bcl-XL can be delivered to specific target cells via cell surface receptors to prevent cell death in vitro and in vivo. Chimeric proteins, containing Bcl-XL fused to the receptor-binding domain of different bacterial toxins (8, 9) or to the transduction domain of the human immunodeficiency virus Tat protein (10, 11), rescue neurons in vivo from axotomy (9), ischemia (10–12), and trauma-induced cell death (13). These observations suggest that the systemic delivery of Bcl-XL could be a clinically relevant strategy to prevent pathological apoptosis.

Granulocyte macrophage-colony stimulating factor (GM-CSF) was originally discovered by the capacity to stimulate both granulocyte and macrophage colony growth from precursor cells in mouse bone marrow (14). It has been shown that this cytokine has other functions mainly arising from its ability to affect the cell number and the activation state of more mature cells such as granulocytes, macrophages, and eosinophils particularly during immune and inflammatory reactions. The functions of GM-CSF are mediated by binding to a specific receptor comprised of a GM-CSF specific α chain and, in humans, a signal transducing β subunit, which is shared with IL-3 and IL-5 receptors (15–17). GM-CSF receptors are found in tissues derived from hematopoietic cells as well as in other cell types including those in the nervous system, such as astrocytes, oligodendrocytes, bone marrow-derived microglia, and to a lesser extent on neurons (18).

The predominant clinical use of GM-CSF is to accelerate marrow recovery after cancer chemotherapy and considerable interest has focused on the use of GM-CSF in stem cell transplantation either for peripheral blood mobilization of stem cells to allow peripheral blood stem cell collection or after autologous stem cell transplantation to decrease the duration of neutropenia (19). GM-CSF also induces destruction of tumor cells...
in vitro by stimulating peripheral blood monocytes (20), enhances dendritic cell maturation (21, 22), and has become an important component of certain vaccine trials (23). Augmentation of stem cell expansion could increase the supply of cells for dendritic cell vaccines for cancer immunotherapy (22).

To further enhance cell survival by reducing apoptosis in cells expressing GM-CSF receptors, we fused the gene of the human growth factor, GM-CSF, to the gene for the human pro-survival protein Bcl-XL. We find that a chimeric protein comprised of GM-CSF and Bcl-XL significantly extends monocyte survival beyond the activity of GM-CSF and may afford an improved treatment for monocytopenia.

**EXPERIMENTAL PROCEDURES**

**Construction of the GM-CSF-Bcl-XL Plasmid**—The GM-CSF cDNA digested with NdeI and BamHI (New England Biolab, Beverly, MA) was ligated with the cDNA of human Bcl-XL digested with BglII and EcoRI. The fused cDNA was then cloned into the plasmid pET28b(+) (Novagen, Madison, WI) digested with Ndel and EcoRI.

**Protein Expression**—Escherichia coli BL21 DE3 (strain OneShot® BL21DE3, Invitrogen) was used to express the GM-CSF-Bcl-XL fusion protein. Recombinant bacteria (transformed with the expression plasmid pET28+ containing the cDNA for GM-CSF-Bcl-XL) were grown in 1L of Super Broth (3.2% tryptone, 2.0% yeast extract, 0.5% NaCl, pH 7.5, KD Medical, Columbia, MD) containing 50 μg/ml ampicillin (Sigma) in 2-liter flasks at 37 °C. Protein expression was induced by addition of 1 mM of isopropyl β-D-thiogalactopyranoside (Sigma) when the A<sub>600</sub> reached 0.8–1 OD. After 3 h incubation, cells were harvested by centrifugation at 5,000 × g, and after resuspension in His tag binding buffer (5 mM imidazole, 20 mM Tris/Cl, pH 7.9, 0.5 M NaCl), pellets were lysed by French press. The inclusion bodies with cellular debris were collected by centrifugation at 5000 × g and washed four times with 20 ml of binding buffer. The supernatant from the final centrifugation was removed, and the inclusion bodies were dissolved in 30 ml of binding buffer containing 6 M guanidine HCl. After incubation on ice and the inclusion bodies were dissolved in 30 ml of binding extract and washed with 5 volumes of binding buffer containing 6 M guanidine HCl. The eluted protein was reduced with 25 mM dithiothreitol and refolded by dropwise dilution in a 100-fold volume of the refolding buffer (0.1 M Tris/Cl, pH 8.0, 0.5 M arginine, 1 mM oxidized glutathione) followed by incubation at 25 °C for 48–72 h. The protein was concentrated with an Amicon Ultra 15 MWCO 10,000 (Millipore, Bedford MA) until a concentration ≥1 mg/ml and dialyzed against PBS. The quality of purified proteins was analyzed by 4–20% SDS-PAGE stained with Brilliant Blue R and Western blotting using a His tag primary antibody (Novagen). The concentration of GM-CSF-Bcl-XL was determined by a colorimetric assay (BCA kit, Pierce). Final yield of GM-CSF-Bcl-XL was between 2–5 mg/liter of culture. The protein was sterilized by filtration through a 0.22 micron membrane and was stored at 4 °C.

**Binding Assay**—To determine the binding affinity of unlabeled proteins relative to that of GM-CSF, HL60 cells were plated in 96-well plates in binding buffer (RPMI containing 1% bovine serum albumin) and incubated with <sup>125</sup>I-GM-CSF 10 nM with and without different concentrations of unlabeled proteins. After 30 min in ice, the cells were centrifuged and washed with binding buffer and then counted.

**Cell Lines and Cell Viability Assay**—HL-60, HeLa, A431, and J774A1 cell lines were purchased from the American Type Culture Collection (ATCC). Buffy coats and apheresis of normal healthy donors were obtained from the National Institutes of Health Blood Bank. Peripheral blood mononuclear cells were isolated on Ficoll gradients. The peripheral blood mononuclear cells were resuspended in RPMI, 10% fetal calf serum (Biofluids, Rockvile MD) and incubated for 2 h in tissue culture dishes 150 × 25 mm. The medium, which contains nonadherent cells, was removed, and the cells were washed two times with complete RPMI. The adherent monocytes/macrophages were gently scraped and centrifuged. Monocyte/macrophage cells were incubated at concentrations of 1 × 10<sup>5</sup> cells/ml in 96-well microtiter plates, overnight, and treated with various concentrations of purified proteins for the required time in Iscove’s modified Dulbecco’s medium, 20% fetal calf serum, 10 ng/ml IL-3, 10 ng/ml IL-6, 10 ng/ml G-CSF.

To access the biological activity of the recombinant protein, different kinds of assay were performed. Cell viability was determined with the Celltiter 96 Aqueous One Solution Cell Proliferation assay kit (Promega, Madison WI). The number of viable cells was determined by quantitation of the ATP present, which signals the presence of metabolically active cells. The amount of ATP was measured as luminescence produced by the mono-oxygenation of luciferin catalyzed by the Ultra-Glo-luciferase.

Calculation of apoptotic cells was performed using the ApoOne Homogeneous Caspase 3/7 Assay kit (Promega) and by fluorescence-activated cell sorting (FACS) analysis. The caspase 3/7 protease activity was measured as fluorescent intensity subsequent to the cleavage of the substrate bis-N-carbobenzoxy-DEVD-rhodamine 110. For FACS analysis, HL-60 cells (1 × 10<sup>6</sup> cells/sample) were labeled with Annexin V-FITC (Apoptosis Detection kit, Trevigen), Hoechst 33342, and propidium iodide (PI) (Vybrant Apoposis Assay kit #5, Invitrogen), according to the manufacturer’s specifications. The cells were analyzed using a dual laser FACS Vantage SE flow cytometer (BD Biosciences). Annexin V-FITC and PI signals were excited using a 488-nm laser light and their emissions captured using bandpass filters set at 530 ± 30 and 613 ± 20 nm, respectively. Hoechst 33342 was excited using a 351-nm UV laser light and its emission captured with a bandpass filter set at 450 ± 20 nm. Cell Quest Acquisition and Analysis software (BD Biosciences) was used to acquire and quantify the fluorescence signal intensities and to graph the data as bivariate dot density plots. Fluorescence emissions of individual fluorophores were corrected for spectral overlap using electronic compensation.

**Bcl-XL Chimera for Cell Protection**
Human CD34+ hematopoietic progenitors from a single donor (Stem Cell Technologies) were seeded at 600 cells/dish for the measurement of burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte/macrophage (CFU-GM) in complete or incomplete methylcellulose-based medium (Stem Cell Technologies). The components of the complete methylcellulose medium were: 1.0% methylcellulose in Iscove’s modified Dulbecco’s medium, 30% fetal bovine serum, 1% bovine serum Albumin, 10−4 M 2-Mercaptoethanol, 2 mM l-glutamine, 50 ng/ml rh stem cell factor, 10 ng/ml GM-CSF, 10 ng/ml IL-3, and 3 units/ml erythropoietin. The incomplete methylcellulose medium does not contain the added cytokines and growth factors.

RESULTS

Expression of GM-CSF-Bcl-XL—In an attempt to prevent death of cells of the myeloid lineage, we designed a GM-CSF-Bcl-XL fusion protein that may be able to deliver Bcl-XL into cells to inhibit apoptosis. The cDNA of human Bcl-XL was fused to the 3′ end of the cDNA for human GM-CSF (Fig. 1A). GM-CSF-Bcl-XL protein expressed in E. coli was insoluble and found in inclusion bodies. After denaturation and purification of the His-tagged protein by affinity chromatography, the protein was refolded by dilution in the presence of glutathione and arginine. The resulting protein was 90% homogeneous and had the expected molecular weight as shown by SDS-PAGE and Western blot (Fig. 1B, lane 1). To confirm the folding of the recombinant fusion protein for the GM-CSF receptor, we tested its ability to bind HL-60 cells and to displace radiolabeled GM-CSF. As shown in Fig. 1C, the EC50 of GM-CSF-Bcl-XL, the concentration necessary for 50% competition of 125I-GM-CSF, was 1.54 nM. Although the affinity is 73 times less than the affinity of the cytokine alone (EC50 = 0.021), the calculated value is similar to the values found for other chimeric proteins containing human GM-CSF as the receptor-binding domain and in the nanomolar range (24).

To compare expression, efficacy, and the importance of the C-terminal membrane anchor of Bcl-XL, a construct, GM-CSF-Bcl-XLΔC, carrying Bcl-XL(1–209), lacking the C-terminal 24 amino acids, fused to the C terminus of GM-
GM-CSF, was also cloned, expressed in *E. coli* and purified (Fig. 1B, lane 2).

**GM-CSF-Bcl-XL Stimulates HL-60 Proliferation**—We tested the biological activities of the fusion proteins on a myeloid cell line expressing the GM-CSF receptor at the concentration corresponding to that of another GM-CSF chimera at its highest biological activity (25).

As shown in the top panel of Fig. 1D, GM-CSF-Bcl-XL stimulated HL-60 cell proliferation to a greater extent than GM-CSF alone. After 48 h there was a 30% increase in cell number in the presence of the chimera, relative to control cells, perhaps due to an inhibition of a background cell death rate in the cell culture. GM-CSF-Bcl-XL extended cell survival in the presence of the broad specificity kinase inhibitor, staurosporine (sts), from 24 h until at least 72 h. After 48-h incubation in the presence of GM-CSF-Bcl-XL and sts, the number of cells is similar to the number of control cells incubated with PBS and after 72 h only 20% of the cells died compared with 50% in the untreated population. In contrast, GM-CSF is not able to block the cytotoxic effect of sts although the concentration is largely in excess with respect to the GM-CSF-binding sites (24).

We also found that the GM-CSF-Bcl-XL chimera protected HL-60 cells from apoptosis induced by sts (Fig. 1D, bottom) as the caspase activity in cells treated with GM-CSF-Bcl-XL was less than cells treated with either GM-CSF or with PBS (Fig. 1D).

The prosurvival activity of GM-CSF-Bcl-XLΔC lacking the C-terminal tail was comparable with that of the chimera of GM-CSF with full-length Bcl-XL (Fig. 1D). This is similar to another Bcl-XL chimeric protein (9) indicating that the C terminus of Bcl-XL is not essential for the prosurvival activity. Since the yield of GM-CSF-Bcl-XL was higher than the yield of GM-CSF-Bcl-XLΔC, we used GM-CSF-Bcl-XL for the subsequent experiments.

**Antia apoptotic Effect of GM-CSF-Bcl-XL**—An early and ubiquitous event in apoptosis is the flipping of phosphatidylserine from the inner to the outer side of the plasma membrane lipid bilayer allowing its detection by the phosphatidyl-binding protein Annexin V. DNA fragmentation distinctive of apoptotic cells can be detected by staining with a nucleic acid dyes such as the fluorophore Hoechst 33342. Both Annexin V and Hoechst 33342 can be detected by staining with a nucleic acid dyes such as the fluorophore Hoechst 33342. Both Annexin V and Hoechst 33342 can be used in combination with PI that stains cells with compromised membrane integrity. PI is excluded from early apoptotic cells but enters into cells with an increased membrane permeability occurring at the later stages of apoptosis or during necrosis. Thus, populations of viable, early apoptotic, late apoptotic, and necrotic cells can be accurately distinguished and quantified by FACS analysis based on their different staining patterns (26).

GM-CSF-Bcl-XL protected HL-60 cells from apoptosis induced with sts (Fig. 2C) much better than GM-CSF alone (Fig. 2B). The viable cell population negative for Annexin staining (R9), decreased 80% as early, middle, late apoptotic (R10, R3, R5, R6) and necrotic (R7) cell populations increased upon incubation with sts for 4 h (Fig. 2A and D). GM-CSF increased the viable cell population about 10% (Fig. 2, B and D), whereas incubation with GM-CSF-Bcl-XL increased viable cells by more than 50% (Fig. 2, C and D).

Similar results were found by visually counting cells positive for Hoechst and PI. Cell viability of 30% in control cells exposed to 0.1 μM sts for 24 h increased to 60% upon addition of GM-CSF-Bcl-XL (data not shown).

**Specificity of the Fusion Protein**—We determined the effect of GM-CSF-Bcl-XL on two human cell lines not expressing the GM-CSF receptor and on a mouse cell line expressing the GM-CSF receptor, since it is known that murine GM-CSF receptor does not detectably bind human GM-CSF (27). When GM-CSF-Bcl-XL was added to GM-CSF receptor negative cells or to cells expressing the mouse receptor neither enhancement of proliferation nor protection from apoptosis were detected, in contrast to the human monocytic cell line positive for the GM-CSF receptor (Fig. 3A), indicating that the binding to the human GM-CSF receptor is required for cell protection activity of GM-CSF-Bcl-XL.

To assess the role of internalization of the chimeric protein on bioactivity, we inhibited an early endosome compartment of the endocytic pathway. We incubated HL-60 cells with NH_4Cl that raises the endosomal pH by reducing the proton gradient across the membrane and with bafilomycin A that is a specific inhibitor of the endosomal V-ATPase proton pump. We used a concentration of the two compounds that does not cause significant apoptosis activation (Fig. 3B). Both bafilomycin and NH_4Cl blocked the activity of GM-CSF-Bcl-XL indicating that the chimeric protein is specifically bound and internalized by the GM-CSF receptor and requires endosomal acidification to mediate apoptosis inhibition.

**Effect of Bcl-XL on Monocyte/Macrophage Populations**—To determine whether the results obtained with HL-60 cells are valid for normal human hematopoietic cells, monocytes from normal donor peripheral blood were incubated with GM-CSF-Bcl-XL in the presence of sts. Although sts was first described as an inhibitor of protein C kinase, it has become clear that it is a broad specificity inhibitor of a diverse array of different kinases (28). We, therefore, assessed the importance of the Bcl-XL portion in the fusion protein prosurvival activity, using another kinase inhibitor inhibitor, tyrphostin AG490 (AgTyr490). High affinity binding of GM-CSF to its receptor induces activation of the receptor-associated Jak2 kinase by means of transphosphorylation of the kinase after oligomerization of the receptor subunits. AgTyr490 specifically inhibits the activation of Jak2, blocking leukemic cell growth in *vitro* and *in vivo* (29, 30). We incubated mononocytic cells with different concentrations of GM-CSF-Bcl-XL in the presence of AgTyr490 or sts for 72 h. As shown in Fig. 4A, the prosurvival effect of an excess of GM-CSF alone is partly inhibited by sts and completely inhibited by AgTyr490. In contrast, GM-CSF-Bcl-XL protects from both kinase inhibitors in a dose-dependent manner. At 0.24 μM GM-CSF-Bcl-XL, slightly less than the 0.36 μM concentration of GM-CSF used in this experiment, a 40 and 30% increase in cell viability was measured in the presence of sts and AgTyr490, respectively, whereas GM-CSF increased cell survival 20 and 0%. We also tested another Bcl-XL fusion protein, Lfn-Bcl-XL (9), that inhibits apoptosis in neurons and should function independently of the GM-CSF receptor. Like GM-CSF-Bcl-XL and in a similar dose range (0.7 μM), Lfn-Bcl-XL inhibited apoptosis of peripheral blood mononuclear cells treated with either

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sts or AgTyr490. Thus, Bcl-XL fusion proteins can protect monocytes/macrophages from apoptosis even when the pro-survival pathway activated by GM-CSF is inhibited.

Cytarabine/AraC and daunorubicin, which have been used for the treatment of leukemias and solid tumors, induce apoptosis (31–34). However, apoptosis of normal myeloid cells results in one of the major side effects of treatment, myelosuppression. Thus, we determined whether GM-CSF-Bcl-XL would inhibit chemotherapy induced apoptosis. Cytarabine/AraC and daunorubicin induce apoptosis of monocytes as measured by caspase 3/7 activity (Fig. 4B). GM-CSF-Bcl-XL is able to reduce the caspase 3/7 activation in monocytes treated with either of these cytotoxic chemotherapeutic drugs and the inhibition is more efficient than GM-CSF applied at a slightly higher concentration (Fig. 4B). The decrease in the catalytic activity of caspase 3/7 is dose-dependent and a concentration of

FIGURE 2. The anti-apoptotic activity of GM-CSF-Bcl-XL. Human promyeloblast HL-60 cells were incubated in the presence of PBS (A), 0.36 μM human GM-CSF (B), and 0.36 μM GM-CSF-Bcl-XL (C). After 24 h, sts at 1 μM was added for 4 h. Immediately thereafter, cells were harvested and simultaneously stained for Hoescht 33342, PI, and Annexin V, A, fluorescence signal intensities of 100,000 cells were quantified by FACS and the conjoint signals of Hoescht 33342 and PI, and Annexin V and PI stainings were displayed in a pseudocolored dot density plot. The results shown in each panel are representative of three independent cell cultures. Cells in R2, with normal Hoescht and no PI, were defined as viable cells; those in R3, with high Hoescht and no PI, were defined as early apoptotic cells with condensed DNA; those in R4, with normal Hoescht and low PI, were defined as early apoptotic/early necrotic cells; those in R5, with low Hoescht and low PI, were defined as middle apoptotic cells; those in R6, with low Hoescht and high PI, were defined as late apoptotic and cell in R7, with normal Hoescht and high PI, were defined as necrotic cells. Cells in R8, with no Hoescht and no PI, were defined as debris. Cells in R9, Annexin V negative and no PI, were defined as total non-apoptotic cells. Cells in R10, Annexin V-positive and no PI, were defined as early apoptotic cells. Cells in R11, Annexin V-positive and low PI, were defined as mid/necrotic cells. Cells in R12, Annexin V-positive and high PI, were defined as late/necrotic cells. Cells in R13, Annexin V-negative and low PI, were defined as cells with loss of membrane integrity. Cells in R14, Annexin V-negative and high PI, were defined as dead/late necrotic/cytotoxic cells (26). D, summary of FACS analysis considering the percent of total viable cells (R9), the percent of the total apoptotic cells (R10 + R3 + R5 + R6) and the percent of the necrotic cells (R7). The error bars represent the standard deviation of the mean of triplicate determinations. Statistical comparisons between treatments marked as a through e in Fig. 2D were significant at p < 0.001 (two-tailed t test).
GM-CSF-Bcl-XL of 2.4 μM reduces caspase activity about 50% (Fig. 4C).

We conclude that GM-CSF-Bcl-XL increases cell viability in cells treated with cytotoxic agents by apoptosis inhibition. GM-CSF-Bcl-XL combines two activities, the GM-CSF kinase activation activity and the Bcl-XL apoptosis inhibition activity to offer a unique approach for myeloprotection.

**GM-CSF-Bcl-XL and CD34**

—We evaluated the effect of GM-CSF-Bcl-XL on hematopoiesis in methylcellulose semi-solid medium colony assays. CD34 cells are GM-CSF receptor positive pluripotent cells having both the capacity for self-renewal and the ability of sustained differentiation into all mature hematopoietic lineages. The commitment is determined by the subsequent expression of lineage-restricted antigens and the presence of specific growth factors (35).

When the CD34 cells from human bone marrow were plated in medium supplemented with the stem cell factor (SCF), erythropoietin, and cytokines specific for myeloid and erythroid differentiation, GM-CSF-Bcl-XL increased the total number of colonies 2-fold (CFU-GM and BFU-E) (Fig. 5A). The growth of the CFU-GM and BFU-E colonies was drastically impaired by cytarabine. Interestingly, incubation of the CD34 cells with GM-CSF-Bcl-XL selectively protected the CFU-GM colonies relative to the BFU-E colonies (Fig. 5A), consistent with the GM-CSF receptor mediating delivery of the Bcl-XL in a cell type-selective manner. Deprivation of cytokines caused a complete loss of CFU-GM and BFU-E colonies but GM-CSF-Bcl-XL protected the myeloid precursors from cytokine deprivation. The activity of the fusion protein protected myeloid progenitors from cytokine deprivation and from the cytotoxic effect of cytarabine, stimulating the differentiation only of the precursors of the monocyte/macrophage lineage (Fig. 5B). From these data, it appears that both GM-CSF and Bcl-XL domains contribute to the proliferation/protection activity, whereas the differentiation effect can be attributed to the GM-CSF portion.

CD34 bone marrow cells cultured in the presence of Lfn-Bcl-XL, which targets Bcl-XL to cells through either the tumor endothelial marker 8 or capillary morphogenesis protein 2 receptors, protects cells from the cytotoxic effect of cytarabine but does not induce growth or differentiation in essential medium (Fig. 6). Under the conditions of growth factor/cytokine deprivation, no colonies were found in wells containing Lfn-Bcl-XL (data not shown). However, in growth factor supplemented medium, Lfn-Bcl-XL increased the number of progenitor colonies without any significant difference in activity between BFU-E and CFU-GM compared with cells incubated with PBS (Fig. 5). Thus, in contrast to GM-CSF-Bcl-XL, Lfn-Bcl-XL lacked the specificity of CFU-GM relative to BFU-E and lacked the growth factor-mediated growth stimulation. Therefore, GM-CSF-Bcl-XL combines two complementary growth promoting activities that function by distinct mechanisms in one recombinant fusion protein.
DISCUSSION

Excessive apoptosis can be deleterious in a number of human diseases such as neurodegenerative disorders, stroke, AIDS, and myelosuppression as a major side effect after chemotherapy (36). Hematopoietic cells are exceptionally prone to apoptosis due to their continual and rapid turnover making the regulation of their rate of cell death particularly important. In the last decade Bcl-2 family fusion proteins that can enter cells have been produced that promote neuron survival. These constructs contain anti-apoptotic Bcl-2 family proteins fused to cell-binding and/or entry domains. In mice, delivery of a Tat protein linked to Bcl-XL significantly reduced brain injury due to a variety of insults (10–12) and Bcl-XL, fused to a portion of the receptor-binding domain of the anthrax toxin (lethal factor) that mediates cell binding and entry inhibited axotomy-induced apoptosis of retinal ganglion cells after optic nerve transaction (9). Lfn-Bcl-XL also increased survival of dorsal horn neurons and ventral horn motoneurons after experimental spinal cord injury in rats (13). Thus, delivery of Bcl-2 family proteins can prevent apoptosis in vivo.

We have created a new chimeric protein by fusing a growth factor, GM-CSF, to target human myeloid cells through the GM-CSF receptor, to Bcl-XL that prevents hematopoietic cell apoptosis during treatment with the cytotoxic drugs utilized in chemotherapy. This fusion protein is the first completely human derived prosurvival chimeric protein reported and is therefore expected to elicit a minimal immunogenic response by the patient compared with proteins constructed with viral or bacterial derived domains. Recombinant GM-CSF-Bcl-XL binds monocytes/macrophages expressing the GM-CSF receptor and delivers Bcl-XL into the cells where it blocks cell death. Its efficacy in apoptosis prevention is higher than the action of GM-CSF alone and the protein remains active due to the activity of the Bcl-XL portion even in the presence of specific drugs that inhibit prosurvival signals from the GM-CSF receptor. Indeed, the functional activity of the Bcl-XL portion is demonstrated as well by a similar increase in cell

FIGURE 4. The effect of GM-CSF-Bcl-XL on human blood mononuclear cells. A, macrophage/monocytes purified by adhesion from monocyte apherensis were treated with human GM-CSF 0.36 μM, different concentrations of GM-CSF-Bcl-XL, and Lfn-Bcl-XL (0.7 μM), a chimeric protein constituted of the binding domain of the anthrax lethal factor and human Bcl-XL, plus the anthrax protective antigen (28 μg/ml) in the presence or absence ofsts (0.1 μM) and the Jak2 kinase inhibitor TyrAg-490 (0.5 μM), for 72 h. Cell viability was determined as described in the legend to Fig. 1. B, caspase 3/7 activity of monocyte/macrophages was measured as described above in the presence of cytotoxic drugs, 0.1 μM cytarabine and 0.1 μM daunorubicin, after 48 h. C, the cells were incubated in the presence of GM-CSF-Bcl-XL at different concentrations for 48 h. The mean values for the experiments in A–C were calculated from five different experiments and each point in each experiment was determined from triplicate sample. The values are presented as a percentage relative to PBS-treated cells. Differences in the mean values marked as * between GM-CSF-Bcl-XL-treated cells and PBS-treated cells (Fig. 4, A–C) were statistically significant at \( p < 0.001 \) (two-tailed t test).
viability in the presence of kinase inhibitors caused by GM-CSF-Bcl-XL compared with Bcl-XL fused to the receptor-binding domain of the anthrax toxin, Lfn-Bcl-XL.

GM-CSF-Bcl-XL protects CD34+ progenitor cells from growth factor deprivation and chemotherapy-induced apoptosis. In a medium formulated to support optimal growth of erythroid progenitors and granulocyte-macrophage progenitors, the GM-CSF domain of the chimera drives the maturation of CD34+ precursors toward the monocyte lineage and the Bcl-XL domain rescues the cells from death induced by cytotoxic drugs. The GM-CSF portion is responsible for maturation, because in the absence of growth factors, only CFU-GM colonies survive.

It was demonstrated that Bcl-XL is poorly expressed in renewing primary erythroblasts, whereas it is strongly up-regulated in maturing erythroid cells in a strictly Epo-dependent manner. Retroviral transduction of Bcl-XL in erythroid progenitors did not alter their dependence on renewal factors such Epo, SCF, and dexamethasone during sustained proliferation but allowed the cells to undergo terminal erythropoiesis in the complete absence of Epo (37). Essentially Bcl-XL protects the erythrocyte progenitors from apoptosis allowing them to complete their differentiation. Similarly, the GM-CSF portion of the GM-CSF-Bcl-XL fusion protein dictates cellular maturation and Bcl-XL protects the progenitor cells. Therefore, even in absence of cytarabine when the CD34+ cells are treated with GM-CSF-Bcl-XL in medium without SCF and erythropoietin, erythroblasts are not derived from the CD34+ progenitors, only monocytes/macrophages. Bcl-XL appears to have no influence on the developmental pathway and simply protects the appropriate lineage from apoptosis. Similar results have been observed in neuronal differentiation pathways exposed to Lfn-Bcl-XL.3

We can conclude that the fusion protein technology is an efficient and promising approach to carry proteins to cells and, in particular, since the GM-CSF receptor is expressed on specific cell types, delivery of Bcl-XL through this binding domain may yield a therapeutically relevant approach to regulating cell viability and preventing or reducing consequences of pathological apoptotic processes. Furthermore, we have produced a prosurvival chimeric protein that is derived solely of human sequences and combines both the prosurvival and differentiation activities of the parent domains.

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FIGURE 5. Hematopoietic colony assay in the presence of GM-CSF-Bcl-XL. CD34+ cells were plated in supplemented (A) or essential medium (B) and incubated for 14 days with different concentrations of GM-CSF-Bcl-XL or in the presence of 0.1 µM cytarabine (right panels). CFU-GM and BFU-E colonies were counted. Results represent the average colony number from three different experiments where each point in each experiment was determined in triplicate. Only differences marked as * between CFU-GM colonies from GM-CSF-Bcl-XL treated cells and PBS treated cells or between BFU-E colonies from GM-CSF-Bcl-XL-treated cells and PBS-treated cells were statistically significant at p < 0.001 (two-tailed t test). Differences between BFU-E and CFU-GM colonies from GM-CSF-Bcl-XL-treated cells were statistically significant at p < 0.001 (two-tailed t test) where marked as **. Cultures with cells alone or with PBS were used to set the value for control cell growth. Control cultures with cells in medium or in medium plus PBS were used to set the value for normal growth.

FIGURE 6. Hematopoietic colony assay in the presence of Lfn-Bcl-XL. CD34+ cells were plated in supplemented medium or essential medium and incubated for 14 days with different concentrations of Lfn-Bcl-XL or in presence of 0.1 µM cytarabine (right panel). CFU-GM and BFU-E colonies were found only with supplemented medium, and they were counted. Results represent the average colony number from three different experiments where each point in each experiment was determined in triplicate. Only differences marked as * between BFU-E colonies from Lfn-Bcl-XL-treated cells and PBS-treated cells or between CFU-GM colonies from Lfn-Bcl-XL-treated cells and PBS-treated cells were statistically significant at p < 0.001 (two-tailed t test). Differences in the mean values between BFU-E and CFU-GM colonies from Lfn-Bcl-XL treated cells were not statistically significant at p < 0.001 (two-tailed t test). Cultures with cells alone or with PBS were used to set the value for control cell growth. Control cultures with cells in medium or in medium plus PBS were used to set the value for normal growth.
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