Fas Antigen Expression on CD34+ Human Marrow Cells Is Induced by Interferon γ and Tumor Necrosis Factor α and Potentiates Cytokine-Mediated Hematopoietic Suppression In Vitro

By Jaroslaw Maciejewski, Carmine Selleri, Stacie Anderson, and Neal S. Young

Activation of Fas antigen, a cell surface receptor molecule, by its ligand results in transduction of a signal for cell death. The Fas system has been implicated in target cell recognition, clonal development of immune effector cells, and termination of the cellular immune response. Fas antigen expression on lymphocytes is regulated by interferon γ (IFNγ) and tumor necrosis factor α (TNFα), cytokines that also have inhibitory effects on hematopoiesis. We investigated Fas antigen expression on human marrow cells and the effects of Fas activation on hematopoiesis in vitro. Freshly isolated immature hematopoietic cells, as defined by the CD34 marker, did not express Fas antigen at levels detectable by fluorescent staining. CD34+ cells, which include progenitors and stem cells, showed low levels of Fas expression in culture, even in the presence of growth factors. Stimulation by TNFα and IFNγ markedly increased Fas antigen expression on CD34+ cells. Anti-Fas antibody, which mimics the action of the putative ligand, enhanced IFNγ- and TNFα-mediated suppression of colony formation by bone marrow (BM) in a dose-dependent manner. This effect did not require the presence of accessory cells. Colony formation from mature (CD34+CD38−) and immature (CD34+CD38+) progenitor cells and long-term culture initiating cells were susceptible to the inhibitory action of anti-Fas antibody in the presence of IFNγ and TNFα. Apoptosis assays performed on total BM cells and CD34+ cells showed that anti-Fas antibody induced programmed cell death of CD34+ BM cells. Fas antigen may be expressed as part of the differentiation program of hematopoietic cells. Fas antigen and its ligand may play a role in the pathophysiology of marrow failure states and in the elimination of abnormal hematopoietic cells in the course of an immune response.

We speculated that the inhibitory effects of TNFα and IFNγ on hematopoiesis might be mediated or potentiated by the Fas receptor/Fas ligand system. We investigated both Fas expression on hematopoietic cells and the consequences of activation of this receptor on marrow cell proliferation in vitro.

MATERIALS AND METHODS

BM cell preparation. BM was obtained from healthy volunteers by aspiration from the posterior iliac crest into syringes containing media supplemented 1:10 with heparin (O’Neill and Feldman, St Louis, MO). Informed consent was obtained according to a protocol approved by the Institutional Review Board of the National Heart, Lung and Blood Institute (Bethesda, MD). Mononuclear BM cells were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). After washing in Hanks’ balanced salt solution (HBSS; GIBCO, Gaithersburg, MD), cells were resuspended in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) supplemented with 20% fetal calf serum (FCS; GIBCO).

Separation of CD34+ cells. CD34+ cells were separated using affinity column (Cellpro, Bathell, WA) and microfluorometry. Briefly, nonadherent BM cells were incubated at room temperature with murine antihuman CD34 IgM, washed in phosphate-buffered saline (PBS), followed by incubation with streptavidin-conjugated goat F(ab’)2 antirat IgM. After washing with PBS supplemented with 2% human albumin, cells were applied to an affinity column

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containing biotin-coated beads, and the CD34⁺ cell fraction was eluted with PBS. An aliquot of the eluted cells was stained with phycoerythrin (PE)-conjugated anti-CD34 HPLC-2 MoAb (Becton Dickinson, Mountain View, CA) to assess the purity of the eluted cells. Usually, 70% to 90% of separated cells were CD34⁺. For higher purity preparations, cells were further fractionated: column-purified cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD34 MoAb and PE-conjugated anti-CD38 MoAb (Becton Dickinson), washed with PBS, and sorted by microcytometry (Epics V; Coulter, Hialeah FL). The purity of cells obtained by combining affinity chromatography and flow cytometry was 97% to 99%.

**Flow cytometric analysis.** FITC-conjugated F(ab')₂ fragments of a murine antihuman Fas IgG1 MoAb (clone UB2; Amac, Westbrook, ME) were used to determine the expression of Fas receptor on BM cells. In some experiments, a PE-conjugated mouse antihuman CD95 IgG1 MoAb was used (clone DX2; Pharmingen, San Diego, CA). Generally similar results were obtained with these two antibodies; brighter staining was seen with PE-conjugate, but the FITC-labeled F(ab')₂ fragments were more specific. For two-color analysis, PE-conjugated MoAb to CD34, CD33, CD3, CD56, CD14, and CD13 (Becton Dickinson) were used in combination with FITC-conjugated anti-CD95 MoAb. Appropriate isotypic controls were used in all experiments.

**Hematopoietic cell culture.** For short-term suspension cultures, CD34⁺ BM cells were cultured in 96 round-bottom well plates at a density of 5 × 10⁵ cells/mL (1 × 10⁵ cells/well) in media consisting of IMDM, 20% FCS, 50 ng/mL interleukin-3 (IL-3; Genzyme, Boston, MA), 50 ng/mL stem cell factor (SCF; Amgen, Thousand Oaks, CA), and 2 U/mL erythropoietin (EPO; Amgen). TNFα, IFNγ, and anti-Fas MoAb were added at appropriate concentrations. After 5 weeks of culture, nonadherent cells were procured using trypsin solution (GIBCO), washed, and replated in methylcellulose to estimate the numbers of long-term culture initiating cells (LTCIC). The number of clonogenic cells was converted to the absolute number of LTCIC by dividing by 4.⁴⁴,⁴⁶

Hematopoietic progenitors were measured in methylcellulose cultures. Freshly isolated or previously cultured BM cells were plated in methylcellulose in the presence of 50 ng/mL IL-3 (Genzyme, Boston MA), 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Boehringer), 50 ng/mL stem cell factor (SCF), and 2 U/mL EPO. Total BM cells were plated at a density of 1 × 10⁶ cells in 1 mL of culture medium in 35-mm dishes. CD34⁺ cells were plated at a density of 1 × 10⁵ cells/0.5 mL methylcellulose in 48-well, 11-mm plates. TNFα, IFNγ, and anti-Fas MoAb were added to the culture at appropriate concentrations. All cultures were performed in duplicate. Experimental procedures were performed in endotoxin-free plasticware, and levels of endotoxin contamination of cytokine preparations were <0.13 endotoxin U/mg by the limulus assay, according to the manufacturers’ specifications. Antibodies used in all experiments contained less than 1 ng/mL of endotoxin. For control experiments for flow cytometry and tissue culture experiments, isotypic MoAb were used (Dako, Carpenteria CA).

**Apoptosis assays.** DNA fragmentation was measured after extraction of low molecular-weight DNA. Two × 10⁷ cells were suspended in 900 μL × TRIS-EDTA buffer and lysed with 25 μL 20% sodium dodecyl sulfate.⁴⁷ High-molecular-weight DNA was precipitated for 6 hours in the presence of 5 mol/L NaCl. The high molecular-weight fraction was pelleted by high-speed centrifugation, and the fragmented DNA was extracted from the aqueous phase as protomers. TNFα, IFNγ, and anti-Fas MoAb were added weekly at appropriate concentrations. After 5 weeks of culture, nonadherent cells were procured using trypsin solution (GIBCO), washed, and replated in methylcellulose to estimate the numbers of long-term culture initiating cells (LTCIC). The number of clonogenic cells was converted to the absolute number of LTCIC by dividing by 4.⁴⁴,⁴⁶

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Fig 2. Dose-dependent induction of CD95 expression on purified CD34+ cells. CD34+ cells were cultured in the presence of IFNγ or TNFα for 48 hours and stained as described in the legend to Fig 1. The proportion of CD34+ cells CD95+ was calculated by dividing the percentage of CD34+CD95+ by the percentage of CD34+ cells in culture.

with phenol and chloroform and precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ethidium bromide staining.

To quantitate the number of cells undergoing apoptosis, cells were fixed with 4% neutral buffered formalin and cytocentrifuged onto siliconized slides. Apoptotic cells were stained using the terminal deoxynucleotidyl transferase method (ApopTag; Oncor, Gaithersburg MD). Endogenous peroxidase was first quenched with 0.5% hydrogen peroxide and the cells were permeabilized using company-supplied equilibration buffer. The 3'-OH ends of degraded DNA were reacted with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled adenosine triphosphate for 30 minutes. After washing with PBS, slides were reacted with an anti-digoxigenin MoAb conjugated to peroxidase, washed and developed with 3,3' diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL). Stained cells were counted using a light microscope.

RESULTS

Expression of Fas antigen (CD95) on BM cells. We first determined whether fresh BM cells expressed Fas antigen (CD95). CD95 expression in total mononuclear BM cells was measured using a combination of FITC-conjugated MoAb to Fas (CD95) and PE-labeled MoAbs to lymphocytes (CD3), natural killer cells (CD56), myeloid cells (CD33, CD13), monocytes, and macrophages (CD14). In freshly isolated BM, within the lymphocyte population, 25% of CD3+ and 3% of CD56+ cells displayed CD95 antigen. Only low percentages of CD95+ cells were detected among myeloid cells (CD13, CD33), monocytes and macrophages (CD14), and in the CD34+ population that contains hematopoietic progenitor cells. Fas antigen expression increased slightly when total BM cells were cultured in the presence of hematopoietic growth factors (data not shown). Upon stimulation with IFNγ, expression of Fas antigen increased on total BM. Both TNFα and IFNγ also increased the percentage of CD33+ cells expressing Fas receptor on myeloid cells (Table 1).

Freshly isolated, purified CD34+ cells also showed low Fas expression, and CD34+ cells, cultured either in the absence of added cytokines or in the presence of a cocktail of hematopoietic growth factors (SCF, IL-3, and EPO) showed only modest expression of Fas antigen. A large proportion of CD95+ cells (and increased CD95-FITC mean log fluo-
Table 2. Effect of Anti-Fas MoAb on Hematopoietic Colony Formation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anti-Fas</th>
<th>Control</th>
<th>Anti-Fas</th>
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</thead>
<tbody>
<tr>
<td>BM Ery (%)</td>
<td>100</td>
<td>100</td>
<td>85 ± 3</td>
<td>113 ± 11</td>
</tr>
<tr>
<td>BM Myelo (%)</td>
<td>100</td>
<td>100</td>
<td>21 ± 11</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>CD34+ Cells Ery (%)</td>
<td>59 ± 9</td>
<td>64 ± 8</td>
<td>44 ± 6</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>CD34+ Cells Myelo (%)</td>
<td>75 ± 2</td>
<td>68 ± 8</td>
<td>48 ± 11</td>
<td>54 ± 11</td>
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</table>

Values represent percentage ± SD of control samples. A total of two experiments were performed with total BM cells and four experiments with purified CD34+ cells. In each experiment, all cultures were performed in duplicate. Mean absolute number of colonies in the controls was 308 ± 24 erythroid and 125 ± 13 for myeloid colonies (per 10^6 cells) for total BM and 57 ± 3 erythroid and 38 ± 7 myeloid colonies (per 10^6 cells) for purified CD34+ cells. TNFα was used at the concentration of 10 ng/mL and IFNγ at the concentration of 1,000 U/mL. Anti-Fas MoAb was used at the concentration of 0.5 µg/mL.

Abbreviation: ery, erythroid; myelo, myeloid.

rescence intensity) were detected when purified CD34+ cells were cultured in the presence of TNFα or IFNγ or the two cytokines together (Fig 1, Table 1). Similar results were observed for CD34+ cells when total BM cells were cultured in the absence of growth factors; IFNγ and TNFα also increased Fas expression on CD34+ in these cultures with or without added hematopoietic growth factors (data not shown).

The expression of CD95 on CD34+ cells was maximal at 48 hours of culture and required concentrations of TNFα ≈ 10 ng/mL and IFNγ ≈ 1,000 U/mL. Higher concentrations of these factors resulted in major loss of cell viability; at the lower concentrations used, cell death, as determined by trypan blue exclusion, was <15%. The strongest induction of Fas antigen expression was observed in the presence of both IFNγ and TNFα (Table 1), but observations over a wide range of IFNγ and TNFα concentrations suggested an additive rather than synergistic effect (Fig 2).

**Inhibition of hematopoietic colony formation by anti-Fas MoAb.** As IFNγ and TNFα stimulated expression of CD95 on CD34+ BM cells, we tested whether treatment with a specific MoAb to Fas antigen, known to mimic the action of natural Fas ligand,1-14 would influence hematopoietic colony formation by total BM cells. Total erythroid and myeloid colonies were determined after 14 days in standard clonogenic assays. Anti-Fas MoAb alone had minimal effect on colony formation (Fig 3). When either total BM cells or purified CD34+ cells were cultured in the presence of antibody and concentrations of TNFα or IFNγ known to result in approximately 50% inhibition of colony formation, anti-Fas antibody greatly potentiated these inhibitory effects (Fig 3; Table 2). A second anti-Fas MoAb, which blocks Fas ligand activation,15 did not potentiate the action of IFNγ or TNFα (Fig 3). Activating Fas antibody–mediated inhibition of colony formation was dose dependent as measured by inhibition of colony formation by CD34+ cells in the presence of suboptimal concentrations of IFNγ and TNFα (Fig 4). Binding of Fas antigen by the activating MoAb enhanced inhibition of colony formation by very low concentrations of IFNγ (20 U/mL) and TNFα (0.2 ng/mL), which ordinarily do not result in significant hematopoietic suppression in vitro (Fig 4).
Table 3. Effects of Anti-Fas MoAb on Generation and Maintenance of LTCICs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Anti-Fas</th>
<th>IFNγ</th>
<th>IFNγ + Anti-Fas</th>
<th>TNFa</th>
<th>TNFa + Anti-Fas</th>
</tr>
</thead>
<tbody>
<tr>
<td>From total BM cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTCIC/10^6 cells</td>
<td>11 ± 2</td>
<td>11 ± 3</td>
<td>8 ± 2</td>
<td>3 ± 2</td>
<td>7 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>LTCIC/culture</td>
<td>69 ± 4</td>
<td>69 ± 5</td>
<td>20 ± 3</td>
<td>9 ± 3</td>
<td>22 ± 5</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>From CD34⁺ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cultured on allogeneic stroma</td>
<td>26 ± 5</td>
<td>20 ± 3</td>
<td>5 ± 2</td>
<td>1 ± 1</td>
<td>9 ± 3</td>
<td>5 ± 4</td>
</tr>
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Values represent mean ± SD of two independent experiments. IFNγ and TNFa were added to the culture weekly at concentrations of 1,000 U/mL and 10 ng/mL, respectively. Anti-Fas MoAb was used at 0.5 μg/mL. Statistical analysis (paired t-test): BM cells: control versus IFNγ, P < .01; control versus TNFa, P < .01; IFNγ versus IFNγ + anti-Fas, P < .01; TNFa versus TNFa + anti-Fas, P < .01; CD34⁺ cells: control versus IFNγ, P < .01; control versus TNFa, P < .01; IFNγ versus IFNγ + anti-Fas, P < .05; TNFa versus TNFa + anti-Fas, P < .05.

We also tested whether the effect of anti-Fas MoAb was limited to a more mature subpopulation of CD34⁺ cells, as defined by the expression of the CD38 antigen.⁴⁷ Equivalent enhancement of the inhibitory action of IFNγ and TNFa by anti-Fas MoAb was observed for both committed CD34⁺CD38⁺ cells and more immature CD34⁺CD38⁻ cells, and again only a modest effect of anti-Fas MoAb alone was observed for cultures not treated with TNFa or IFNγ (data not shown).

**Effect of anti-Fas MoAb on LTCICs.** Because anti-Fas antibody enhanced the inhibition by IFNγ and TNFa of colony formation from CD34⁺ cells, we tested whether very immature hematopoietic cells, defined by their ability to form colonies after 5 weeks of suspension culture (also referred to as LTCIC), were suppressed by triggering of Fas-receptor. In long-term cultures, activating anti-Fas MoAb alone did not affect the generation or maintenance of LTCIC; however, it much augmented the negative effects of IFNγ and TNFa on both the generation of LTCIC and on stroma formation in LTBMCE (Table 3). Similar results were obtained when highly purified CD34⁺ cells were seeded onto preformed irradiated allogeneic stroma and cultured for 5 weeks under LTBMCE conditions. Anti-Fas MoAb alone showed only a modest effect on LTCIC generation but potentiated the inhibitory effects of IFNγ and TNFa on the numbers of LTCIC generated (Table 3).

**Ability of anti-Fas MoAb to mediate apoptosis of BM cells in culture.** Triggering of the Fas receptor pathway induces apoptosis of lymphoid cells.⁵⁻⁷ Enhanced inhibition of colony formation by anti-Fas MoAb also might be caused by induction of cell death of clonogenic progenitors. To test this hypothesis, we stimulated total BM cells with IFNγ and TNFa (at suboptimal concentrations that induced minimal apoptosis) in the presence and absence of anti-Fas antibody. Low molecular-weight DNA extracted from constant numbers of these cells cultured with anti-Fas MoAb showed a nucleosomal degradation pattern typical of apoptosis (Fig 5A). To determine whether anti-Fas MoAb-mediated apoptosis of hematopoietic progenitor cells, we visualized the nucleosomal ladder by electrophoresis of low molecular weight DNA extracted from purified CD34⁺ cells (10⁶ cells per extraction) stimulated with IFNγ or TNFa (Fig 5B). We also measured the number of apoptotic cells in cultures of CD34⁺ cells stimulated with anti-Fas MoAb in the presence of these cytokines using an in situ assay (Fig 5C). Anti-Fas MoAb increased the number of CD34⁺ cells in apoptosis in cultures containing negative cytokines.

**DISCUSSION**

We have detected Fas antigen on human hematopoietic cells cultured in vitro. Most of our studies were performed with a purified population of marrow cells selected for ex-
pression of the CD34 antigen; this population does not contain lymphocytes but includes hematopoietic progenitor and stem cells.

Although freshly isolated marrow cells showed low levels of Fas antigen expression on their cell surface, culture of marrow in the presence of growth factors led to a moderate increase in Fas expression, predominantly in differentiated cells. These results are perhaps analogous to the induction of Fas expression in the immune system. Fas antigen expression is low on most resting rodent and human lymphocytes but is induced by activation of T and B cells with lectin, antigen, or growth factors like IL-2.

Fas antigen expression was not high on fresh or cultured CD34+ cells, a more primitive population that includes progenitor and stem cells. Similar to immune cells, Fas antigen expression also increased in the presence of TNFα; previously, downregulation of the TNF receptor and Fas antigen have been shown to be coregulated. Fas antigen expression was associated with profound functional effects on hematopoietic cell proliferation and viability. Activation of Fas markedly reduced both progenitor cell-derived colony formation from CD34+ cells and the generation of LTCICs in long-term culture or from CD34+, in the presence of inhibitory cytokines. The effect of anti-Fas MoAb was dose-dependent and synergistic with IFNγ and TNFα, best shown by the dramatic effect of MoAb to Fas on colony formation at suboptimal concentrations of these cytokines.

As with lymphocytes, on hematopoietic cells Fas antigen stimulation was correlated with the induction of apoptosis in the CD34+ population. Thus, induction of Fas antigen expression by TNFα and IFNγ would render the cells susceptible to Fas ligand- (or anti-Fas MoAb)-mediated apoptosis. This cooperative action of inhibitory cytokines may account for their synergistic inhibitory effect on hematopoietic cell proliferation in vitro. Suppression of colony formation likely results in this setting at least in part because of the killing of clonogenic progenitors. Our results, of course, do not exclude other mechanisms of apoptotic cell death independent of Fas antigen stimulation. Indeed, we observed a more profound effect of anti-Fas MoAb on colony formation than on CD34+ cell viability. Fas might have been selectively induced on colony-forming cells, induced on progeny cells during differentiation in vitro or, alternatively, cell proliferation was suppressed independent of cell death. Thus, hematopoietic suppression after stimulation of Fas may be caused by mechanisms other than apoptosis, such as metabolic disturbances or a block in cell cycle progression. Recently, bcl-2 has been shown to prevent Fas-mediated cell death, and upregulation of this protein because of growth factor stimulation might protect hematopoietic progenitors from killing, even if they were unable to enter cell cycle. Whether Fas represents a final pathway for programmed cell death induced by inhibitory cytokines or Fas ligand cooperates synergistically with IFNγ and TNFα is the subject of current experiments.

In the presence of anti-Fas MoAb and either IFNγ or TNFα, the very marked reduction in hematopoiesis in vitro, as observed in direct clonogenic assays and in the LTCIC system extended from mature progenitor cells to cells considered surrogates for true stem cells. Although extrapolation from tissue culture to disease pathophysiology should be undertaken cautiously, cooperation among several coregulated inhibitory cytokines—IFNγ, TNFα, and Fas ligand—would exert a potent negative effect on marrow cells in the setting of immune-system attack. As with IFNγ and TNFα, expression of Fas has been associated with viral infection (on the lymphocytes of patients with adult T-cell leukemia) on cells from children infected with the human immunodeficiency virus, and in the liver of patients with chronic hepatitis C infection. Viral infections are associated with marrow suppression, and viruses have been implicated in hematologic syndromes like aplastic anemia (AA). The genes for IFNγ and TNFα are overexpressed in marrow and blood cells of patients with AA. TNFα mRNA is present in normal BM, in which case limited Fas expression might be implicated in normal hematopoietic cell regulation. If Fas were mainly induced by IFNγ in vivo, the Fas system might be dysregulated in immune-mediated marrow disease. Increased Fas expression and decreased viability of CD34+ cells has been reported in refractory anemia. Interference with the Fas antigen/Fas ligand system might be an attractive target of experimental therapy in marrow failure syndromes.

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