Fas Ligand Is Present in Human Erythroid Colony-Forming Cells and Interacts With Fas Induced by Interferon γ to Produce Erythroid Cell Apoptosis

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Interferon γ (IFNγ) inhibits the growth and differentiation of highly purified human erythroid colony-forming cells (ECFCs) and induces erythroblast apoptosis. These effects are dose- and time-dependent. Because the cell surface receptor known as Fas (APO-1; CD95) triggers programmed cell death after activation by its ligand and because incubation of human ECFCs with IFNγ produces apoptosis, we have investigated the expression and function of Fas and Fas ligand (FasL) in highly purified human ECFCs before and after incubation with IFNγ in vitro. Only a small percentage of normal human ECFCs express Fas and this is present at a low level as detected by Northern blotting for the Fas mRNA and flow cytometric analysis of Fas protein using a specific mouse monoclonal antibody. The addition of IFNγ markedly increased the percentage of cells expressing Fas on the surface of the ECFCs as well as the intensity of Fas expression. Fas mRNA was increased by 6 hours, whereas Fas antigen on the cell surface increased by 24 hours, with a plateau at 72 hours. This increase correlated with the inhibitory effect of IFNγ on ECFC proliferation. CH-11 anti-Fas antibody, which mimics the action of the natural FasL, greatly enhanced IFNγ-mediated suppression of cell growth and production of apoptosis, indicating that Fas is functional. Expression of FasL was also demonstrated in normal ECFCs by reverse transcriptase-polymerase chain reaction and flow cytometric analysis with specific monoclonal antibody. FasL was constitutively expressed among erythroid progenitors as they matured from day 5 to day 8 and IFNγ treatment did not change this expression. Apoptosis induced by IFNγ was greatly reduced by the NOK-2 antihuman FasL antibody and an engineered soluble FasL receptor, Fas-Fc, suggesting that Fas-FasL interactions among the ECFCs produce the erythroid inhibitory effects and apoptosis initiated by IFNγ.

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MATERIALS AND METHODS

Purification and expansion of human blood ECFCs. Four hundred milliliters of blood was obtained from normal donors after informed consent approved by the Vanderbilt University and Department of Medicine/Hematology, Vanderbilt University Medical School MRB II Room 547, Nashville, TN 37232-6305.

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Veterans Affairs Medical Centers (Nashville, TN) Institutional Review Boards. BFU-E were purified by sequential density gradient centrifugation; depletion of platelets, lymphocytes, and adherent cells; and further negative selection of contaminant cells with CD2, CD11b, CD16, and CD45 monoclonal antibodies (MoAbs), as previously described.8,19 The BFU-E were suspended in 20 mL Iscove’s modified Dulbecco’s medium (IMDM) containing 20% heat-inactivated fetal calf serum, 5% heat-inactivated, pooled, human AB serum, 1% deionized bovine serum albumin (BSA; Intergen Co, Purchase, NY), 5 × 10⁻³ mol/L 2-mercaptoethanol, 10 µg/mL insulin, 2 U/mL recombinant human (rh) EP, 50 U/mL interleukin-3, penicillin at 500 U/mL, and streptomycin at 40 µg/mL in 50 mL polystyrene flasks to generate ECFCs.19 After incubation at 37°C in 5% CO₂/95% humidified air for 4 or 5 days (day-5 or day-6 cells), the cells were collected, further enriched by centrifugation through 10% BSA and over Ficoll-Hypaque, and aliquoted for Northern and flow cytometric analyses plus plasma clot assays for ECFCs. In some experiments, incubation was continued in liquid medium with or without rhIFNγ (4.75 × 10⁻² U/mg; Genzyme Corp, Cambridge, MA) for additional times to observe later effects of rhIFNγ. Plasma clot assay for ECFCs. Cells were plated at a concentration of 10³/mL in an IMDM mixture containing 20% fetal calf serum, 5% human AB serum, 1% deionized BSA, 2 U/mL rhEP, penicillin plus streptomycin, 2 mg/mL fibrinogen (Sigma, St Louis, MO), and 0.2 U/mL bovine thrombin (Parke-Davis, Morris Plains, NJ) in 48-well flat-bottomed tissue culture plates with 0.2 mL/well. In some experiments, rhIFNγ; anti-Fas antibodies, CH-11 (Immunotech Inc, Westbrook, ME), and/or ZB4 (MBL, Watertown, MA); anti-FasL antibodies, CH-11 (Immunotech Inc, Westbrook, ME), and/or ZB4 (MBL, Watertown, MA); anti-FasL antibodies, CH-11 (Immunotech Inc, Westbrook, ME), and/or ZB4 (MBL, Watertown, MA); and the appropriate duration of incubation with day-5 cells to produce its inhibitory effects. Inhibition of ECFC growth by IFNγ. To study the effect of IFNγ on ECFCs, we used indirect murine IgG1 stained with FITC-goat anti-mouse IgG1 (Calbiochem, San Diego, CA) or murine IgG1 at the same concentration for 30 minutes. These cells were then washed once with PBS/1% BSA. Secondary incubations were performed with FITC-goat antimouse IgG1 (10 µg/mL; Southern Biotechnology Assoc Inc, Birmingham, AL) for another 30 minutes. To ensure that the Fas⁺ cells were ECFCs, dual staining was performed in some experiments combining indirect immunofluorescence for FasL with direct staining using phycoerythrin (PE)-MoAb (5 µg/mL) to CD71 (transferrin receptor; Caltag). After indirect staining as described above, unbound goat antimouse binding sites were blocked with 5 µg/mL normal mouse IgG in PBS for 20 minutes. Direct-labeled, antigen-specific PE-MoAb to CD71 or PE-murine IgG1 (Becton Dickinson) was then incubated with the cells for an additional 30 minutes. The cells were then fixed and analyzed by flow cytometry. Cells were incubated with murine IgG1, indirectly stained with FITC-goat antimouse IgG1, and counterstained with direct-labeled, nonspecific PE-murine IgG1 to show nonspecific fluorescence on both axes. For specific comparison of murine antihuman FasL MoAb on ECFCs, we used indirect murine IgG1 stained with FITC-goat antimouse IgG1 and counterstained with specific PE-MoAb to CD71. To detect soluble FasL in the cell medium, the sFas Ligand ELISA Kit purchased from MBL was used.

RESULTS

Inhibition of ECFC growth by IFNγ. To study the effect of IFNγ on ECFCs, we first defined the optimum dose of rhIFNγ and the appropriate duration of incubation with day-5 cells to produce its inhibitory effects. Inhibition of ECFC growth was produced by an rhIFNγ concentration as low as 50 U/mL (Fig 1A) and was clearly evident after 72 hours of incubation with rhIFNγ (Fig 1B). Cytospin preparations of these cells after 96 hours of incubation with 1,000 U/mL of rhIFNγ showed cells with morphologic changes characteristic of apoptosis, such as nuclear condensation, fragmentation, and reduced size, which have correlated with DNA fragmentation.
demonstrated by in situ end-labeling in ECFCs. A marked reduction of the number and size of erythroid colonies and hemoglobin formation was also induced by rhIFN-γ, and programmed cell death within the erythroid colonies was enhanced as the concentration of rhIFN-γ was increased (Fig 2). Thirty percent of erythroid colonies contained apoptotic cells after incubation with 50 U/mL of rhIFN-γ and 50% of erythroid colonies were greatly reduced in size and had an enhanced number of cells with the characteristic morphologic changes of apoptosis at the highest concentration of rhIFN-γ. Similar results were obtained in day-6 cells (data not shown).

Expression of Fas on ECFCs. We next determined whether IFN-γ treatment induced Fas mRNA expression. Day-5 ECFCs were cultured with rhIFN-γ at a variety of increasing concentrations and durations of incubation. The cells were then harvested and Northern analysis for Fas was performed (Fig 3). A very low level of Fas mRNA was detected among the original cells without incubation. After incubation with rhIFN-γ, expression of Fas mRNA was markedly enhanced. A greater than fivefold enhancement of Fas mRNA by laser scanning densitometry was evident at an rhIFN-γ concentration of 500 U/mL or higher. This induction of Fas mRNA expression by rhIFN-γ was clearly increased by 6 hours after incubation with rhIFN-γ, and Fas mRNA continued to increase as the time of treatment was extended.

Fas antigen expression on the cell surface was measured by direct immuno-fluorescence with specific FITC-MoAb and flow cytometry. Only a small percentage of Fas+ cells was present among the original cells before incubation, but a very large increase of Fas+ cells was demonstrated after 48 hours of incubation with increasing concentrations of rhIFN-γ (Fig 4A). An enhanced expression of Fas antigen on the cells, including both the number of positive cells and the intensity per cell, was clearly apparent at 24 hours and reached a plateau after 48 to 72 hours of incubation with rhIFN-γ (Fig 4B). No effect was evident at 6 hours (data not shown). An enhanced concentration of Fas on the surface may be required for inhibition of cell proliferation and induction of apoptosis, because a significant reduction in cell number occurred at 72 hours when the cells had a higher Fas distribution on their surface.

FasL expression in ECFCs. Experiments were performed first to demonstrate FasL mRNA by RT-PCR. These studies showed that FasL mRNA was present in cells treated or not treated with rhIFN-γ (Fig 5). To investigate the expression of FasL protein in ECFCs, day-5 cells and their descendant day-8 cells, derived from the day-5 cells by incubation with or without rhIFN-γ for 72 hours, were fixed and permeabilized for identification of FasL and CD71 with specific MoAb. Although all proliferating cells including activated T and B cells and macrophages also bear CD71, the number of transferrin receptors in erythroid progenitor cells is 15-fold higher than the concentrations associated with other cells. The high-intensity CD71+ cells were also larger cells than those with low-intensity CD71+ and ECFCs are large cells. Therefore, when flow cytometric analysis for FasL was performed, large cells with...
high intensity CD71$^+$ were gated. This group of cells represented more than 80% of the total population. To exclude interference due to staining with two fluorescence markers, not only isotype, normal mouse FITC-IgG with mouse PE-IgG was set as a control, but also mouse FITC-IgG with PE-MoAb to CD71 was used as a second control.

Approximately 50% of the gated CD71$^+$ cells expressed FasL by two-color flow cytometric analysis (Fig 6). Figure 6 compares FasL expression on all day-5 CD71$^+$ cells (Fig 6A) and high-intensity CD71$^+$ cells (Fig 6B). All CD71$^+$ cells were 47% FasL$^+$, whereas CD71$^+$ bright cells were 64% FasL$^+$. Plasma clot assay showed that at least 65% ± 6% of the cells were ECFCs. This indicated that most of the FasL$^+$ cells were ECFCs. FasL was constitutively expressed in the erythroid progenitors from day 5 to day 8 (Table 1) and was not altered in day-8 cells after 72 hours of incubation with 1,000 U/mL of rhIFN$\gamma$ (data not shown). When nonpermeabilized cells were used in these experiments, FasL was present on the cell surface in 13% ± 16% of the ECFCs. Day-8 cell culture media from five separate experiments showed an increase in soluble FasL of 83 ± 41 pg/mL compared with undetectable levels in day-8

Fig 3. Dose- and time-dependent induction of Fas mRNA in ECFCs treated with rhIFN$\gamma$. Day-5 cells were incubated with IFN$\gamma$ at 0 to 2,000 U/mL for 72 hours or with 2,000 U/mL for 0 to 72 hours, and then the total RNA was prepared. Twenty micrograms of total RNA was loaded in each lane. The blots were hybridized with a labeled probe for Fas and rehybridized with a probe for actin after stripping the initial probe. The ECFC purity at day 5 was 53% ± 9% and 56% ± 8%, respectively, and by day 8 it was 89% ± 6% and 91% ± 8%.

Fig 4. Expression of Fas on ECFCs after incubation with rhIFN$\gamma$. Day-5 cells were cultured in liquid medium in the presence of rhIFN$\gamma$ at 0 to 1,000 U/mL for 48 hours (A) or at 1,000 U/mL for 24 to 96 hours (B). At the indicated times, the cells were incubated with FITC-MoAb to Fas (CD95) or FITC-murine IgG1. Flow cytometric analysis was then performed. The open histogram shows the CD95 fluorescence of the cells incubated without rhIFN$\gamma$ and the solid histogram shows the CD95 fluorescence of the rhIFN$\gamma$-treated cells. The purity of the day-7 cells was 89% ± 9% (A) and 81% ± 7% (B).
cells were CD71 among these cells, 100% were CD71 clots to obtain the ECFC number among the total cells. Aliquots of day-5 and day-8 cells were cultured in plasma (experiments no. 2 through 4). In experiment no. 1, only FasL was analyzed for FasL and CD71 by flow cytometry after permeabilization. Procedures with superimposed data: (1) population in lower left quadrant represents fluorescence produced by presence of indirect isotype control plus FITC-goat antimouse IgG1 and murine PE-IgG1; (2) gray represents the fluorescence produced by the presence of indirect isotype control plus FITC-goat antimouse IgG1 and specific PE-MoAb to human CD71; (3) dark black represents fluorescence produced by indirect MoAb to FasL stained with FITC-goat antimouse IgG1 and specific PE-MoAb to CD71. (A) CD71 large cell population, representing 84% of all cells; 99% of the cells were CD71 and 47% were FasL. (B) Large cells bearing high intensity CD71 from the same experiment, representing 79% of all cells. Among these cells, 100% were CD71 and 64% were FasL. The purity of the ECFCs was 65% ± 6%.

media incubated without cells. This concentration of soluble FasL is significantly above that of the normal serum level of 58 ± 35 pg/mL (P < .05).

Demonstration of functional Fas and FasL. To determine whether IFNγ inhibition of cell growth was produced by the interaction between Fas (induced by IFNγ) and FasL, already present in the cells, the results of ligation of murine antihuman Fas MoAb to ECFCs was studied. Two specific MoAbs to Fas (CH-11 IgM), which is known to mimic the action of FasL, and ZB-4 IgG, which blocks the effect of CH-11) were tested.

Dose-response curves for CH-11 and ZB-4 to determine the optimum concentrations for our experiments were performed and these were compared with the isotype-matched murine Igs to ensure that the effects of the antibody were specific and not related to IgM or IgG addition. When CH-11 or ZB-4 were added to day-5 ECFCs, no effect on colony size, colony number, or hemoglobin concentration was apparent (data not shown). Day-5 ECFCs were then cultured in liquid medium in the presence of a concentration of rhIFNγ known to result in a large increase of Fas, and approximately 50% inhibition of the cell number plus an additional 40% inhibition of viability were noted (Fig 7A). The addition of anti-Fas MoAb, CH-11, to the rhIFNγ-treated ECFCs greatly potentiated the reduction of cell number by rhIFNγ, whereas ZB-4 had little effect. When CH-11 and ZB-4 were added together, the effect of CH-11 was blocked by ZB-4. Fas MoAb-mediated inhibition of colony formation was also observed (Fig 7B). CH-11 strongly increased the rhIFNγ inhibitory effect on colony size and number. More than 90% of erythroid colonies were suppressed with the addition of CH-11, even when the concentration of rhIFNγ was quite low (50 U/mL) and ZB-4 neutralized the CH-11 reduction of colony number as well. These data clearly indicated that Fas induced by IFNγ was functional and could be activated.

NOK-2, a specific neutralizing MoAb to human FasL, was used to confirm the functional activity of FasL in these cells. The results from two experiments are shown in Table 2. In the presence of rhIFNγ at 250 U/mL, the first experiment showed that more than two thirds of the colonies had morphologic apoptotic changes and less than one third looked normal. Erythroid colony size and colony number were greatly reduced. The addition of NOK-2 almost completely restored the number of colonies, although colony size remained small. Apoptosis was not evident in the presence of NOK-2. In the second experiment, the donor cells were more sensitive to rhIFNγ and normal colony formation was almost completely inhibited with rhIFNγ. More than 70% of the colony-forming cells were rescued and had normal morphologic characteristics in the presence of NOK-2. These experimental results were confirmed by similar findings with the addition of Fas-Fc, although the latter was not quite as potent (Table 2).

**DISCUSSION**

The inhibitory effect of IFNγ on the ECFCs was observed both in liquid and plasma clot cultures and inhibition of cell proliferation occurred at a concentration of rhIFNγ as low as 50 U/mL. Evidence that this inhibition is a direct effect of rhIFNγ is provided by the observations that the degree of inhibition did not vary significantly as the ECFCs were purified from 19.8% to 52.6%, that ECFCs with a purity of 80% were still inhibited, and that ECFCs have rhIFNγ receptors. This finding is...
without 50 U/mL rhIFN presence of CH-11 (100 ng/mL) and/or ZB4 (500 ng/mL) with or without 50 U/mL rhIFN g1 Fas-Fc 6 rhIFN NOK-2 6 Control 6 Control 6 Control 6 Experiment no. 1 Control 33 ± 2 20 ± 3 14 ± 2 0 rhIFNγ 16 ± 2 40 ± 2 rhIFNγ + NOK-2 2 ± 1 33 ± 3 28 ± 3 0 rhIFNγ + Fas-Fc 14 ± 2 43 ± 2 7 ± 1 Experiment no. 2 Control 20 ± 5 25 ± 7 24 ± 2 0 rhIFNγ 51 ± 4 rhIFNγ + NOK-2 6 ± 3 42 ± 4 13 ± 2 rhIFNγ + Fas-Fc 2 ± 1 27 ± 2 28 ± 2 Day-5 cells were cultured in plasma clots with or without rhIFNγ at 250 U/mL and with or without neutralizing anti-Fasl MoAb, NOK-2, or Fas-Fc. After incubation for 10 days, the clots were fixed and stained. NOK-2 (20 µg/mL) was added at the start of incubation and 2.5 µg/mL was added at 24 hours and 48 hours of incubation. Fas-Fc (10 µg/mL) was added at the start of incubation and 5 µg/mL was added at 24 hours of incubation. Equal volumes of media were added to the controls.

*Erythroid colony number per 100 cells plated; large colonies, greater than 500 cells per colony; medium colonies, 50 to 500 cells per colony; small colonies, 2 to 49 cells per colony.

†Colonies with more than 10% apoptotic cells.
cytometric analysis showed that approximately 50% of the large CD71+ cells, which were 80% of the total cells, expressed FasL. Because not all the cells in the population were ECFCs, especially at day 5, and because erythroid cells have 15-fold more CD71 than other cells, we performed flow cytometric analysis with the cells gated to include only the high-intensity, more CD71 than other cells, we performed flow cytometric especially at day 5, and because erythroid cells have 15-fold

FasL. Because not all the cells in the population were ECFCs, which IFN and EP receptors as well as the mRNA for these receptors. 45

reported. 41 FasL is constitutively expressed in normal thyroid IFN, results in programmed cell death. 43,44 Further studies are now in progress to delineate

ECFC. Our results showed that approximately 66% of the large size, high-intensity, CD71+ erythroid cells were FasL+ and that FasL was constitutively expressed on ECFCs from day 5 to day 8. Because day-8 cells were almost homogenous, with 90% or more of the day 8 cells proven to be ECFCs by plasma clon assay, most of the FasL+ cells in our cultures are ECFCs and rhIFNγ did not appear to alter FasL expression.

Nok-2, an anti-FasL MoAb, has the biological property of neutralizing FasL. 26 When this MoAb was added to the ECFC cultures, the induction of apoptosis and the inhibition of ECFC proliferation and colony formation by IFNγ were partially prevented, indicating that FasL is functional as an actuator of apoptosis in the system. Comparable results were observed with Fas-Fc. Our observations thus suggest a mechanism responsible for at least part of the inhibitory effect of IFNγ on human ECFCs. Because normal ECFCs express negligible amounts of Fas, they do not undergo apoptosis. Simultaneous expression of functional Fas and FasL on ECFCs, the former induced by IFNγ, results in programmed cell death.

Similar findings in Hashimoto’s thyroiditis have now been reported. 41 FasL is constitutively expressed in normal thyroid cells, but only the cells of Hashimoto’s thyroiditis express large amounts of Fas on their cell surface, which is thought to be induced by IL-1β. Coexpression of Fas and FasL also has been thought to contribute to the rapid rate of spontaneous neutrophil apoptosis. 42

Because Fas and FasL were coexpressed in ECFCs after incubation with IFNγ, they may commit the cells to an autocrine death as shown with T-cell hybridoma cells 20 and/or soluble FasL may function in a paracrine pathway to mediate cell death. 5,44 Further studies are now in progress to delineate the precise mechanism of this interaction. The mechanism by which IFNγ exerts an inhibitory effect on the cell growth of ECFCs and induces apoptosis may be multifactorial. Our laboratory has found that IFNγ downregulates stem cell factor and EP receptors as well as the mRNA for these receptors. 45 Nevertheless, the present study shows that the Fas/FasL system strongly contributes to the inhibition of erythropoiesis by IFNγ.

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