CD4+ T-Cell Induction of Fas-Mediated Apoptosis in Burkitt's Lymphoma B Cells

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Cytotoxic function of CD4+ T,0 cells is mediated by Fas (CD95, APO-1) and its ligand (Fas ligand). Recent studies using nontransformed B cells and the Ramos Burkitt's lymphoma (BL) B-cell line cells show that CD40 ligation at the B-cell surface by activated, CD40 ligand (CD40L)-bearing, CD4+ T cells upregulates Fas expression on B cells and primes B cells for Fas-mediated death signals. In this work, we examine whether this CD4+ T-cell-dependent molecular pathway for Fas upregulation and B-cell apoptosis reflects a peculiarity of the Ramos B-cell line cell or is applicable to other Burkitt's tumors as well. In 5 of the 6 Epstein-Barr virus-negative BL cell lines examined, the cells constitutively express undetectable or low levels of Fas and are resistant to Fas-mediated signals induced by mononclonal anti-Fas anti-

A P0PTOTIC DELETION of inappropriately expanded B-cell populations is fundamental in the avoidance of autoimmune disease and in immune regulation of some B-cell malignancies. Fas (CD95, APO-1) is a 48-kD cell surface antigen of the tumor necrosis factor/nerve growth factor (TNF/NGF)-receptor superfamily that can mediate apoptosis of a variety of cell types, including lymphocytes.1,2 Fas ligand is selectively expressed or secreted by activated Th1 CD4+ T cells3 and, when bound to the Fas receptor on the lymphocyte, induces its apoptotic destruction.4,5 Fas-mediated signals are necessary for the physiologic elimination of self-reactive B cells.6 Although cells from a variety of hematologic neoplasms have been shown to express the Fas antigen, there is a dissociation between Fas expression and function. Not all cells that express Fas are sensitive to Fas-mediated signals.7

Most investigations of T-cell-mediated cytotoxicity and tumor control have focused on MHC-I restricted, CD8+ T-cell-mediated cytotoxicity,10,11 but several observations suggest that CD4+ T cells are critical regulators of the normal humoral immune response as well as of aberrant B-cell proliferations.12-15 Small, noncleaved cell lymphoma (SNCL; Burkitt's) is a pathologically distinct subset of B-cell lymphomas that represents the malignant outgrowth of germinal center B cells.16,17 We have shown that activated CD4+ T cells can modulate the growth of Ramos Burkitt's lymphoma (BL) B-cell line cells by ligating CD40 at the B-cell surface, which results in Fas upregulation and confers sensitivity to Fas-mediated death signals.18 The finding that CD40 ligation could result in augmented Fas expression and function19,20,39 was somewhat surprising in the context of CD40's previously known function in transducing a potent B-cell activation, differentiation, and survival signal.20,21 In our laboratory, this observation was largely an outcome of work in which the Ramos cells were used as a model for studying germinal center B-cell activation and function.

To see if CD4+ T-cell mediated Fas upregulation and B-cell death reflects a peculiarity of the Ramos B-cell line cells or is applicable to other BL, we tested this signaling pathway using other Burkitt's cell lines. In addition, we implemented our system using malignant BL cells isolated from the peripheral blood of a patient with chemotherapy-resistant disease. The studies confirm that Epstein-Barr virus (EBV)-negative BL cells from four cell lines and from a refractory clinical tumor can be induced by CD40L+ T cells to express Fas and undergo apoptosis in vitro. These results indicate an important role for CD4+ T cells and Fas-mediated B-cell cytotoxicity in immune modulation of Burkitt's lymphoma.

MATERIALS AND METHODS

Cell culture. Unless otherwise specified, the BL B cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and were cultured in C50 medium (RPMI-1640 medium with penicillin, streptomycin, glutamine, and 10% fetal bovine serum). The BL2, BL31, and BL41 BL lines were the generous gift of Dr David Thorley-Lawson (Tufts University, Boston, MA) and have been described previously.22 These were cultured in C50 supplemented with 2 mmol/L NaPyruvate. Clones of the Jurkat lymphoblastoid T-cell lines, B2.7 and D1.1, were kindly provided by Dr Seth Lederman (Columbia University College of Physicians and Surgeons, New York City, NY). B2.7 is deficient in CD40L expression, whereas clone D1.1 constitutively expresses CD40L.23 Co-culture experiments were performed using irradiated (2,000 rads) Jurkat T cells and target B cells in a ratio of 1:4. The kinetics of Fas upregulation and death in this system have been described previously by our group.24 Briefly, Fas expression on the B cells

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increases over the initial 48 to 72 hours of coculture with CD40L+ T cells, but T-cell-induced, Fas-ligand-mediated death does not occur until 48 to 96 hours. The irradiated T cells used in these experiments are functional in expressing CD40L and induce activation antigen expression on target B cells, but die in culture within 48 hours, such that, by analysis at 48, 72, or 96 hours, CD3+ cells are not detected by flow cytometry.

Purification and analysis of cells from a clinical case. Tumor B cells from the patient were isolated from fresh, heparinized blood and were easily propagated in vitro using media with 10% fetal bovine serum, without the addition of cytokines or other supplements. Flow cytometric analysis of these cells showed a monoclonal population of B lymphocytes that expressed CD10, CD19, CD20, and CD23. Cytogenetic analysis of cells from the bone marrow aspirate identified a typical t(8:14) translocation and additional aneuploid features. After approximately 2 months in culture, the cells appeared and grew in a manner quite similar to those of the Ramos BL cells and a cell line termed “Easter,” was established. Cells analyzed from both early and late cell passages were not detected by flow cytometry.

Flow cytometry. Cells were washed in cold Hank’s Buffered Saline Solution (HBSS) and stained with antibodies according to standard techniques. Stained cell fluorescence was measured using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA) and analyzed with the CellQuest program (Becton Dickinson).

Monoclonal antibodies (MoAbs). Sterile antibodies used in cell culture experiments included antihuman Fas (murine IgM: Upstate Biotechnology Inc, Lake Placid, NY), anti-Fas- (murine IgG; Immunotech, Westbrook, ME), anti-CD23 (murine IgG, EBVCS2, and murine IgM, EBVCS3; ATCC), anti-Fas F(ab')2 (generous gift of Dr Peter H. Krammer, German Cancer Research Center, Heidelberg, Germany), anti-CD40 and anti-CD40L (anti-gp39: Genzyme, Cambridge, MA), and W6/32 (anti-MHC-I; gift of Dr Carlo Russo, Cornell University Medical College, New York, NY). Additional antibodies used for staining before flow cytometry include anti-Fas-fluorescein isothiocyanate (FITC; Immunotech), anti-CD31–FITC (Immunotech), anti-CD19–FITC (Immunotech), anti-CD4–phycoerythrin (PE; Immunotech), anti-CD4–PE (Immunotech), anti-CD3 (OKT3; ATCC), anti-CD10 (Coulter Immunology, Hialeah, FL), anti-CD21 (PharMingen, San Diego, CA), anti-CD30 (BioSource Int, Camarillo, CA), anti-CD54 (anti-ICAM; Immunotech).

Viability assay. Aliquots of cultured cells were diluted 1:4 in 0.4% trypan blue and analyzed for the exclusion of dye by light microscopy.

Flow cytometric cell cycle analysis. After Ficoll-Hypaque centrifugation, purified, viable cells were permeabilized in 70% ethanol at 0°C for 15 minutes, washed 1x in HBSS, and resuspended in HBSS containing propidium iodide at 50 µg/mL with RNase at 5 µg/mL. After incubation at 37°C for 30 minutes, the suspensions were stored in the dark at 4°C until the time of analysis. DNAs were analyzed directly on the Becton Dickinson FACSscan.

AlamarBlue cell proliferation assay. The alamarBlue fluorometric/colorimetric growth indicator (BioSource Int) was used to measure cell proliferation and susceptibility to Fas-mediated killing. Target B-cell line cells that had been exposed in macrowells to media only, to either CD40L+ or CD40L− T cells, or to either monoclonal anti-CD40 or an isotype-matched control MoAb were replated in sterile 96-well round-bottom plates at a uniform concentration and volume. The cells were then exposed to media only, anti-Fas antibody (final concentration, 200 ng/mL), or anti-CD23 antibody (200 ng/mL). After an additional 36 hours, alamarBlue was added (10% by volume) to each well and the relative number of proliferating cells was determined 6 hours later by spectroscopy (excitation, 530 nm; emission, 590 nm) using a CytoFluor 2350 fluorescent plate reader (Millipore Corp, Bedford, MA). For each circumstance, the experiments were performed in triplicate and the relative number of proliferating cells was determined as the mean fluorescence scanning result for each culture circumstance, less the mean obtained for three wells with media alone (background). The results shown represent the mean result obtained from three separate experiments. Standard errors were determined using SigmaPlot (Jandel Scientific, San Rafael, CA).

RESULTS

BL B-cell line cells that constitutively express low levels of Fas upregulate Fas after CD40 ligation. CD40L+ or CD40L− Jurkat T-cell clones were used to effect upregulation of Fas on BL cells from a panel of EBV-negative cell lines. As shown in Fig 1, the constitutive and inducible phenotypes of the lines (BL2, BL31, BL41, ST486, and
CA46) are quite similar to those we have reported with Ramos. In each case, constitutive Fas expression is low to undetectable and Fas expression is upregulated in the presence of the CD40L-bearing T cells, but not in the presence of T cells that are deficient in CD40L. To show that Fas upregulation on the B cell is dependent on the CD40-CD40L interaction, we performed blocking experiments using antibody to CD40L. As shown for the BL41 cells in Fig 2, Fas induction on BL B cells by CD40L+ T cells is specifically inhibited in the presence of antibody to CD40L, but not in the presence of an isotype-matched control antibody.

BL cell apoptosis was observed in 4 of the EBV-negative BL cell lines that were induced to upregulate Fas expression as a consequence of CD40 ligation in the presence of the CD40L-bearing T cells. B-cell death occurred between 48 and 96 hours after exposure to the T cells and was augmented by the addition of anti-Fas MoAb. Anti-Fas antibody is used in vitro to mimic the effects of Fas ligand on Fas-expressing cells. As shown in Fig 3, after CD40 ligation by the T cells, there is an acquired, specific susceptibility to Fas-mediated death signals. For these experiments, after 48 hours of coculture, the target B cells were aliquoted in microwell cytotoxicity plates and exposed either to media alone or to anti-Fas or an isotype-matched control antibody (anti-CD23) at 200 ng/mL. AlamarBlue analysis of the proliferating cell populations after an additional 36 hours showed that specific inhibition of proliferation in response to anti-Fas MoAb occurred only after the cells had been exposed to CD40L+ T cells in coculture. The results indicate that, in all of the EBV-negative BL B-cell lines examined except for ST486, the cells express low levels of Fas and are resistant to Fas-mediated cytotoxicity. In each of these lines except for CA46, the BL cells respond to CD40-mediated signals by upregulating Fas and becoming sensitive to Fas-mediated cytotoxicity. Although there is heterogeneity in the degree of conferred sensitivity among the BL cell lines, it is clear that the capacity to undergo Fas-mediated apoptosis is enhanced in at least 4 of the cases.

**Tumor cells from a resistant Burkitt’s lymphoma/leukemia case undergo Fas-mediated apoptosis after coculture with CD40L+ Jurkat T-cell mutants.** Malignant B cells were isolated from the peripheral blood of a human immunodeficiency virus (HIV)-negative patient with refractory Burkitt’s lymphoma that progressed to a leukemic phase. The patient was a 34-year-old man who presented with odynophagia and a rapidly growing mass at the base of his tongue. Biopsy of the mass, which involved Waldeyer’s ring, showed a monotonous population of cells, with numerous mitotic figures and macrophages, consistent with a small, non-cleaved cell (SNCC; Burkitt’s) lymphoma (Fig 4A). Despite intensive combination chemotherapy, his disease progressed, and within 4 months, the peripheral blood smear showed circulating Burkitt’s lymphoma cells (French-American-British classification L3 leukemia; Fig 4C), and the bone marrow was replaced by malignant lymphoid cells (Fig 4B). The patient died approximately 6 months after the onset of his clinical illness.

The patient’s cells were cultured in the presence of media or with CD40L− or CD40L+ irradiated T-cell clones and then analyzed by immunofluorescence flow cytometry for Fas expression (Fig 5). There was constitutively low Fas expression on the B cells cultured with media alone and with CD40L− T cells. In contrast, after coculture with CD40L+ Jurkat T cells, a significant proportion of this patient’s BL cells expressed augmented levels of the Fas antigen (double positives, right lower panel) and underwent apoptosis (Fig 6a). For this analysis, after 48 hours of coculture, viable cells were isolated by Ficoll-Hypaque centrifugation and then analyzed for DNA fragmentation. Flow cytometric cell cycle analysis after propidium iodide staining showed dramatic augmentation of the subdiploid (apoptotic) DNA only after coculture with the CD40L+ Jurkat T cells. To clarify the mechanism of cell death, in another set of experiments, the patient’s cells were exposed in coculture to the CD40L+ T cells in the presence of a blocking F(ab’), preparation of anti-Fas (Fig 6b). As shown, there is a partial but dose-dependent inhibition of apoptosis in the presence of the
blocking antibody to Fas, whereas there is no effect seen with the control antibody preparation. Taken together, these experiments indicate that the patient's BL cells can be manipulated by ligation of CD40 such that they are rendered susceptible to Fas-mediated cytolysis.

**DISCUSSION**

Epidemiologic data indicate that patients who carry CD4+ T-cell deficiencies have a propensity to develop B-cell non-Hodgkin's lymphoma (NHL), but the precise molecular mechanisms of failed immune surveillance of aberrant B-cell proliferations in that setting have not been established. The data presented in this work provide a link between Fas ligand-expressing cytotoxic CD4+ T cells and Fas-mediated apoptosis of BL cells. We have shown that EBV-negative BL B cells specifically upregulate Fas and become sensitive to Fas-mediated signals after ligation of CD40 at the B-cell surface. This result was obtained in 4 of the 5 BL cell lines that are constitutively resistant to Fas-mediated cytolysis and in malignant cells isolated from a patient with chemotherapy-resistant disease. These results support an immunoregulatory role for CD4+ T cells via the Fas-Fas ligand system and have important implications for both the pathogenesis and therapy of Burkitt's lymphoma.

Once Fas is upregulated in the BL cells, the sensitivity to Fas-mediated cytolysis is variable. It is possible that some resistant tumors carry defects in the Fas receptor itself, such as have been described in one murine B-cell lymphoma or that some bear defects in the distal Fas signaling pathway. Although it is not a factor in the cell lines reported here, EBV may be important in conferring resistance to Fas-mediated cytolysis in EBV-positive BL. Although the role of specific EBV proteins in conferring specific resistance to Fas-mediated apoptosis is unknown, LMP-1 has been shown to bind to a molecule associated with the cytoplasmic domain of CD40 (CRAF-1) and, to a lesser extent, with TNF receptor and Fas-associated cytosolic molecules. This association in the B cell might interfere with either CD40-mediated Fas upregulation or with Fas-mediated death signals. Another possible mechanism of resistance is the direct effect of antigenic stimulation on the malignant or premalignant germinal center B cell. Rothstein et al showed that murine splenic B cells induced to express Fas as a consequence of CD40 ligation can be rescued from Fas-mediated apoptosis by...
**Fig 4.** Histologic analysis of the tumor. (A) Histology of the original biopsy specimen from the lesion at the base of the tongue. There is a starry sky appearance due to a monotonous population of lymphocytes, each with a thin rim of cytoplasm, consistent with SNCC (Burkitt's) lymphoma. Numerous mitotic figures and tingible body macrophages are present. Hematoxylin and eosin (original magnification × 128). (B) Bone marrow aspirate at the time of leukemia, showing normal hematopoietic elements replaced by lymphocytes with large nuclei, open nucleoli, and scant dark blue, vacuolated cytoplasm. Wright-Giemsa stain (original magnification × 330). (C) High-power view of the peripheral blood smear at the time of evolution to leukemia. In the field shown are two typical leukemic cells, consistent with ALL (L3, Burkitt's). Wright's stain (original magnification × 330).

**Fig 5.** Tumor cell death is associated with augmented Fas expression. Two-color flow cytometric analysis of cells cultured with media alone (upper panel); with irradiated, CD40L+ Jurkat T cells (middle panel); and with irradiated, CD40L- Jurkat T cells (lower panel). For each case, contour plots indicate the cells stained with FITC-conjugated anti-Fas (x-axis) and, in the left panel, PE-conjugated anti-CD4 (y-axis) or, in the right panel, PE-conjugated anti-CD19 (y-axis).
cross-linking of the surface antigen receptor. This mechanism could explain some cases of sporadic Burkitt’s lymphoma that, based on Ig sequence analysis, are reported to be antigen-driven.32

Although Burkitt’s lymphoma is not usually ascribed to T-cell defects, the high incidence of this malignancy in patients with HIV disease33,34 suggests that our findings (which implicate CD4+ T cells) have physiologic relevance. Our observations are consistent with the clinical picture of polyclonal B-cell proliferations in HIV,35 a high incidence of autoantibody production,36 and the emergence of uncontrolled clonal expansions. Without HIV, CD4+ aberrations leading to failed Fas upregulation and impaired B-cell apoptosis may be quite subtle and go unrecognized by routine clinical and laboratory parameters. These abnormalities might include inherited, iatrogenic, and infection-associated changes in the CD4+ T-cell population, such as those that occur due to malaria.37

We would emphasize that, in the experiments reported here, the ratio of effector T to target malignant B cells in coculture was 1:4. This suggests that, in sensitive tumor cell populations, a relatively small proportion of cytotoxic T cells can cause death of a substantial B-cell tumor cell population. The fact that BL B cells that constitutively express low or even undetectable Fas levels can be killed by Fas-mediated signals after appropriate T-cell stimulation indicates that the Fas-Fas ligand system may be critical in immune modulation of tumors that are not known to express the Fas antigen. Our data, which show undetectable or low constitutive Fas expression on each BL line examined, are consistent with previous studies that describe BL as a Fas-negative tumor.38 The capacity to manipulate Fas expression may be applicable to other B-cell tumors such as chronic lymphocytic leukemia or follicular non-Hodgkin’s lymphomas, which display resting or inactivated B-cell phenotypes, but which might upregulate Fas in response to appropriate signals and then become sensitive to apoptosis.

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