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Inhibition of Fas-mediated Apoptosis by Antigen: Implications for Lymphomagenesis

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Abstract

Apoptotic deletion of expanded B cell populations is essential in avoidance of autoimmune disease and immune regulation of some B cell malignancies. The role of CD4⁺ T cells in B cell apoptosis is evident from the high incidence of B cell tumors and autoimmunity in patients with T cell diseases such as the acquired immune deficiency syndrome (AIDS). We have previously demonstrated that in Epstein–Barr Virus (EBV) negative Burkitt's lymphoma (BL), a tumor derived from proliferating centroblasts of the germinal center, the malignant lymphocytes can be induced to express Fas (CD95) by ligation of CD40 at the B cell surface. Upon CD40 engagement, BL cells are sensitized to T-cell derived death signals provided by Fas ligand (FasL, CD95L). HBL-3 is a cell line derived from an AIDS-related BL in which the tumor IgM binds the human erythrocyte "i" antigen. To determine whether Fas-mediated apoptosis of BL cells is reduced in the context of antigen to which the tumor IgM binds, we stimulated HBL-3 cells with CD40 ligand (CD40L, CD154) in the presence and absence of human erythrocytes expressing the "i" antigen, and measured Fas-mediated apoptosis upon exposure to an agonistic anti-Fas antibody. We observed that HBL-3 cells were sensitized to Fas-mediated death by exposure to CD40L. When i⁺ RBCs were present, Fas-mediated apoptosis in HBL-3 cells was reduced by greater than 30%. In contrast, there was no reduction in Fas-mediated apoptosis in the presence of i[−] (I[−]) RBCs. These findings demonstrate that Fas-mediated deletion of BL cells is inhibited upon surface IgM engagement by antigen for which the malignant clone has affinity.

Keywords

Burkitt's lymphoma; B lymphocytes; Apoptosis; Fas (CD95); Human immunodeficiency virus (HIV); Immunoglobulin

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INTRODUCTION

Apoptotic deletion of inappropriately expanded B cell populations is fundamental in avoidance of autoimmune disease and in immune regulation of some B cell malignancies. The importance of T cell-mediated apoptosis in tumor regulation is underscored by the high incidence of B cell tumors in patients with T cell diseases.^[1,2] For example, in patients with the acquired immune deficiency syndrome (AIDS), there is a dramatic excess of non-Hodgkin's lymphomas (NHL).^[3–5] Relative to the spectrum of lymphomas seen in patients who are not infected with the human immunodeficiency virus (HIV), the subtypes of lymphoma occurring in HIV disease are disproportionately high-grade tumors.^[2–7] Small non-cleaved cell (SNCL, Burkitt's and Burkitt's-like) tumors represent 25–50% of AIDS-related lymphomas, suggesting a disposition to this particular form of malignancy in patients with HIV.^[6,8,9] In patients with HIV there is also an excess of nonmalignant B cell expansions, manifest by hypergammaglobulinemia and autoimmune humoral blood conditions such as idiopathic thrombocytopenic purpura and autoimmune hemolytic anemia.^[10,11] These clinical observations are consistent with the premise that failed B cell apoptosis in patients with HIV results in some types of B cell lymphoma and autoimmunity.

Burkitt's lymphoma (BL) is one of several tumor types derived from B lymphocytes of the germinal center.^[12–14] The transformed cells display a resting B-cell phenotype characterized by cell surface expression of CD10 (CALLA), CD19 and CD20, CD40, CD77 and IgM.^[13,15,16] Upon biopsy, BL cells usually do not express B cell activation antigens such as CD23, CD86 or Fas (CD95). Distinctive features of this tumor include chromosomal rearrangements resulting in *c-myc* overexpression, and an extraordinarily high proliferation rate with frequent mitoses and cell death.^[17–20] It is established that in some cases of BL and cell lines derived from those, the IgM of the malignant clone has affinity for autologous structures.^[21,22] The role for antigen in the etiology of BL is further supported by sequence analyses of VH and VL genes utilized by the tumors, which demonstrate intraclonal heterogeneity and somatic hypermutation.^[23]

In Epstein–Barr Virus (EBV) negative BL cell lines and in some freshly-isolated tumor specimens, BL cells can be induced to express Fas after they interact with CD40L–expressing T cells.^[24,25] This process of B cell activation confers sensitivity to death signals via Fas ligation, which in the germinal center would be delivered by T cells expressing Fas ligand (FasL, CD95L). Because it is established that antigen receptor cross-linking inhibits apoptosis of activated, Fas-expressing germinal center B cells,^[26,27] we hypothesized that Fas-mediated apoptosis in human BL cells would be reduced in the presence of antigen for which the clone has affinity. To address this question, we utilized HBL-3 cells, derived from an HIV-associated case of BL.^[28] The surface IgM expressed by this tumor has been well-characterized and is known to have high affinity for the human RBC little “i” antigen.^[21] In these studies, we observed that Fas-mediated apoptosis in HBL-3 cells was reduced significantly when i+ RBC were present, but not when the cells were exposed to big “i” (I+) RBC. These results suggest that Fas-mediated deletion of BL cells is reduced when there is binding of antigen to IgM at the B cell surface. The data offer a specific mechanism by which an antigen extrinsic to a malignant lymphoma cell can promote survival of that cell, by reduction of Fas-mediated apoptosis.

METHODS

Cell Culture and Cell Lines

Cells were cultured in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 1% penicillin–streptomycin, 1% L-glutamine (GIBCO), and 20% heat-inactivated fetal calf serum (Gemini Bioproducts, Calabasas, CA) at 5% CO₂ and 37°C. HBL-3 cells have been described previously.^[21,29] Other BL cell lines, used as controls, were the Ramos and HBL-1 cell lines.^[29,30] Clones of the Jurkat lymphoblastoid T cell lines, B2.7 and D1.1, were the kind gift of Dr Seth Lederman (Columbia University College of Physicians and Surgeons, New York City, NY). B2.7 is deficient in CD40L expression, while clone D1.1 constitutively expresses CD40L.^[31] The Jurkat mutants were irradiated (2500 rads) prior to coculture experiments and used in a ratio of 1:4 (Jurkat T: target B cell).

Immunofluorescence Flow Cytometry

Cells were washed in cold Hank's Buffered Saline Solution (HBSS) and examined using monoclonal antibodies according to standard techniques. Cell fluorescence was measured using a FACScan (BD Biosciences, San Jose, CA) and analyzed with the CellQuest program (BD Biosciences).

Monoclonal Antibodies

Antibodies used for indirect immunofluorescence studies included murine anti-human Fas (Beckman Coulter, Brea, CA), anti-CD3 (Beckman Coulter), anti-CD19 (BD PharMingen, San Diego, CA), in each case followed by FITC-conjugated goat anti-mouse Ig. For cytotoxicity assays, we used an agonistic anti-Fas antibody (clone CH11, murine IgM, Upstate Biotechnology Inc., Lake Placid, NY) or, as a control, an isotype-matched antibody to CD23 (EBVCS3, murine IgM, American Type Tissue Collection, Rockville, MD).

AlamarBlue Cell Proliferation Assay

The alamarBlue fluorometric/colorimetric growth indicator (Biosource International, Camarillo, CA) was used to measure cell proliferation and susceptibility to Fas-mediated growth inhibition.^[32–34] HBL cells that had been exposed in macrowells to media only, to CD40L– T cells (clone B2.7) or CD40L+ T cells (clone D1.1), were re-plated in sterile 96-well round-bottom plates at 5×10^4 BL cells in 100l media. The stimulated cells were exposed to media only, to anti-Fas antibody (final concentration 200 ng/ml), or to anti-CD23 antibody (200 ng/ml) and, after an additional 36 h, alamarBlue was added (10% by volume) to each well. The relative number of proliferating cells was determined 6h later by spectroscopy (excitation, 530 nm; emission, 590 nm) using a CytoFluor 2350 fluorescent plate reader (Millipore Corporation, Bedford, MA). These experiments were performed in triplicates, and the relative number of proliferating cells determined as the mean fluorescence scanning result for each culture circumstance, less the mean obtained for three wells with media alone (background).

Analysis of Cellular DNA Content with Propidium Iodide

After purification by Ficoll–Hypaque centrifugation, viable cells were permeabilized in 70% ethanol at 0°C for 15 min, washed once 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 min, the suspensions were stored in the dark at 4°C until the time of analysis. DNAs were analyzed directly using a FACScan and analyzed with CellQuest software.

JAM Assay for DNA Fragmentation

In this assay,^[35] proliferating cells are pulsed with ³H-thymidine for a period of hours (16 h in these experiments), washed, and then exposed to apoptosis effectors such as cytotoxic T cells or anti-Fas antibody. After sufficient time to allow for DNA fragmentation (an additional 24 h), the cells are lysed and harvested using a filter through which intact chromatin will not pass. Upon harvesting, the scintillation result for each circumstance reflects the amount of incorporated ³H-thymidine that was not degraded during apoptosis. The apoptosis percent is calculated as follows:

$$\% \text{ specific DNA loss} = (S - E) / S \times 100,$$

where *S* is the retained DNA in the absence of apoptosis-inducing agent (spontaneous) and *E* is the experimentally-retained DNA.

RBC Experiments

i+ and i– (I+) RBCs were phenotyped and provided by Immucor (Norcross, Georgia). In pilot experiments, i+ RBCs were used at a range of concentrations from 1×10^3 to 1×10^8 RBCs/ml. Once it was established that the optimal dose for apoptosis inhibition is 1×10^4 RBCs/ml (data not shown), RBCs were used in subsequent experiments at a concentration of 1×10^4 RBCs/ml.

RESULTS

HBL-3 Cells are Sensitized to Fas-mediated Growth Inhibition by CD40 Ligation

The HBL-3 cell line was originally derived from the pleural fluid of a patient with a HIV-associated BL.^[21,29] These cells do not contain EBV sequences and demonstrate a typical BL molecular phenotype, including expression of surface IgM (sIgM), MHC Class II, CD10, CD19, CD20 and CD22. As in other EBV negative BL cells,^[25] HBL-3 cells constitutively express Fas at a moderate level but express greater amounts after exposure to irradiated, high CD40L-expressing Jurkat mutants (not shown). Augmented Fas expression in HBL-3 cells upon CD40 engagement increased sensitivity of the cells to growth inhibition by an agonistic antibody to Fas (Fig. 1). In the experiment shown, unstimulated or CD40L-activated HBL-3 cells were exposed to an agonistic anti-Fas antibody, clone CH11. This reagent induces apoptosis in Fas-expressing, Fas-sensitive cells by cross-linking of the Fas receptor at the cell surface. As is evident, although there was some baseline sensitivity of the HBL-3 cells to Fas-mediated growth inhibition, the effect was markedly enhanced after CD40 ligation.

Fas-mediated Apoptosis of HBL-3 Cells is Reduced in the Presence of i+ RBCs

Because the IgM of HBL-3 cells is known to bind the RBC “i” antigen with high affinity,^[21] we examined whether the presence of i+ RBCs affects Fas-mediated apoptosis in HBL-3-cells. For these experiments, HBL-3 cells were induced to express Fas by CD40L+ T cells as we have done previously,^[25] but in the absence or presence of human i+ or i- (I+) RBCs used at 1×10^4 RBCs/ml. Apoptosis was measured by either of two assays. First, we used propidium iodide and immunofluorescence flow cytometry to determine the fraction of cells with subdiploid (apoptotic) DNA content. Figure 2 indicates the results for apoptosis measurement in unstimulated HBL-3 cells, and for HBL-3 cells placed in culture with irradiated CD40L+ T cells, with or without the addition of i+ or i- RBCs. Panel A demonstrates that there is some baseline apoptosis of the HBL-3 cells in culture, which was similar to that we have observed in other BL cell lines.^[25] This degree of ongoing apoptosis is characteristic of BL, and can be ascribed to the high cell turnover rate in this tumor.^[18] Similar to the results of Fig. 1, there was some constitutive sensitivity of HBL-3 cells to Fas-triggered death signals (panel B, 25% subdiploid DNA). However, the effect of Fas ligation was clearly augmented after CD40 ligation in the HBL-3 cells by effector T cells (panel D, 42% subdiploid DNA). We did not observe significant apoptosis among cells exposed to a control, isotype-matched antibody to CD23. As shown in panel E, there was a reduction of the subdiploid DNA fraction after Fas ligation in CD40L-activated HBL-3 cells with i+ RBCs (31% subdiploid DNA), but not in the same cells exposed to i- (I+) RBCs (panel F, 45%). Thus, Fas-mediated apoptosis of the HBL-3 cells was specifically reduced by approximately 33% in the presence of i+ RBCs.

To confirm these results regarding the impact of RBCs on Fas-mediated apoptosis in HBL-3 cells, we utilized an alternative method of apoptosis measurement, the JAM assay for DNA fragmentation (Fig. 3).^[35] The bar graph in panel A (media only) reveals that there was a moderate level of constitutive sensitivity to Fas ligation in unstimulated HBL-3 cells, which is consistent with our previous results. The baseline susceptibility of the HBL-3 cells to Fas-mediated death was not altered significantly by addition of i+ RBCs at the end of the experiment, at the time of Fas cross-linking by antibody (rightmost bars). In contrast, in HBL-3 cells that were primed by CD40 ligation (B–D), there was inhibition of Fas-mediated death in cells exposed to i+ RBCs at the start of HBL-3 activation (D) or after 48 h, at the time of Fas ligation by antibody (B). Similar experiments, using two Burkitt's cell lines (Ramos and HBL-1) that do not bind “i”, did not reveal any impact of i+ RBCs on Fas-mediated apoptosis in the cells (data not shown). Taken together, these results indicate that in the context of activated CD4+ T cells expressing CD40L, Fas-mediated deletion of BL cells is reduced when there is concomitant binding of antigen to IgM at the B cell surface.

DISCUSSION

In these studies we have demonstrated that sensitivity to Fas-mediated death signals in cells from an AIDS-related BL tumor could be induced by interaction of the tumor B cells with CD40L-expressing T cells. Once Fas was induced in the B cells, Fas-mediated apoptosis was reduced specifically in the presence of antigen (i) to which the IgM of the tumor cells binds. The present findings support a mechanism by which antigen in the germinal center,

either autologous or foreign, can promote tumor cell survival. These data indicate that antigen extrinsic to a BL tumor cell, for which the tumor IgM has specific affinity, can inhibit Fas-mediated apoptosis of the tumor (Fig. 4).

The erythrocyte “i” antigen, for which the IgM of the HBL-3 cells has high affinity, is one of two similar carbohydrate structures associated with Band 3 in RBCs. This antigen is typically expressed in fetal but not in adult RBCs.^[36] Occasionally, “i” is expressed in adults, particularly in situations during which the marrow is stressed by conditions such as blood loss, infection, or chemotherapy.^[37,38] Thus, it is possible that in a patient with disease such as HIV, some i+ RBCs are present in the marrow and periphery, and so this antigen would be available to stimulate the malignant, or premalignant clone(s). Anti-“i” IgG and IgM are associated *in vivo* with autoimmune hemolytic anemia,^[38] and have been observed in high titer from patients with lymphoma and Kaposi’s sarcoma.^[39]

Burkitt’s Lymphoma is characterized by rapid proliferation and cell death *in vivo* and *in vitro*.^[17,40] Apoptosis is a common feature of BL cells in biopsy samples. In early histologic and electron microscopic studies of this tumor type, there were identified typical features of apoptosis including cytoplasmic granularity, nuclear blebbing, and cell death.^[17,19,40–42] In recent years, data from our laboratory and others have supported the premise that CD4+ T cells have cytotoxic effects by FasL, and that these cells are key mediators of apoptosis both of normal germinal center B cells and of certain B cell tumors.^[24,25,43–48] In BL, it appears that CD4+ T cells are critical regulators of B cell activation and apoptosis.^[24,25]

In the absence of anti-retroviral treatments, patients with HIV suffer from progressive qualitative and quantitative defects in CD4+ T cells.^[49,50] In North Americans without HIV, BL is a rare B cell malignancy, particularly among adults.^[51] The experiments described in this work support that in some Burkitt’s tumors, clonal expansion occurs due to lack of T cell-modulated, Fas-mediated apoptosis in the B cells. The two classes of NHL that arise most often in AIDS, the Burkitt’s or Burkitt’s-like tumors, and the diffuse large cell B cell lymphomas, each represent outgrowths of germinal center B cells.^[12,13] Thus, there are two distinct impairments which would favor the development of autoimmunity and BL in patients with HIV.^[52] First, the loss of CD4+ T cells would reduce the host’s ability to induce Fas expression and apoptosis in B cells prior to malignant transformation, which might be a factor in autoimmunity in patients with HIV. Second, once a BL tumor has formed, the presence of antigen in the germinal center would inhibit Fas-mediated apoptosis of BL cells specific for that antigen. This mechanism of tumor cell survival due to an exogenous antigen may be relevant in the context of chronic infection, as occurs in patients with HIV. Moreover, inhibition of Fas-mediated apoptosis by autologous structures, such as RBC antigens, would favor survival of malignant BL cells with affinity for those particular antigens.

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Abbreviations

AIDS	acquired immune deficiency syndrome
BL	Burkitt's lymphoma
CD40L	CD40 ligand, CD154
EBV	Epstein–Barr Virus, EBV
Fas	CD95
FasL	Fas ligand, CD95L
HBSS	Hank's buffered saline solution
HIV	Human immunodeficiency virus
Ig	immunoglobulin
RBC	red blood cell

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FIGURE 1.

Sensitivity of HBL-3 cells to Fas-mediated cytotoxicity is enhanced by exposure to CD40L. HBL-3 cells were exposed to media, or to the irradiated CD40L⁻ (clone B2.7) or CD40L⁺ T cell mutants (clone D1.1), and after 48 h of coculture the viable B cells were purified by density centrifugation prior to exposure to an agonistic anti-Fas antibody (clone CH11) at one of three doses. Inhibition of HBL-3 proliferation in response to Fas ligation was determined by reduction of the fluorometric growth indicator alamarBlue.

FIGURE 2.

Fas-mediated apoptosis in HBL-3 cells is inhibited in the presence of i+ RBCs but not i- RBCs. HBL-3 cells were exposed to media alone (histograms A,B), or stimulated by coculture with irradiated CD40L+ T cells in the absence (C,D) or presence of human i+ (E) or i- (F) RBCs at 1×10^4 RBCs/ml. After 48 h, the viable HBL-3 cells were isolated by Ficoll-Hypaque centrifugation, re-aliquoted at 0.5×10^6 cells/ml; and then exposed to media alone (A,C), to an agonistic anti-Fas antibody (B, D-F), or as a control, to an isotype-matched antibody to CD23 (not shown) in the presence or absence of RBCs at 1×10^4 RBCs/ml. After an additional 18 h, the cells were analyzed for DNA content by immunofluorescence flow cytometry. The percent of cells with subdiploid (apoptotic) DNA is indicated for each sample.

FIGURE 3.

Chromatin fragmentation is reduced in CD40-stimulated HBL-3 cells exposed to i+ RBCs prior to Fas engagement. HBL-3 cells were exposed to media (A), or to irradiated CD40L+ T cells in the absence (B) or presence of either i-/I+ (C) or i+ RBCs (D). After 48 h, the cells were pulsed with ^3H -thymidine for 16 h, washed, and exposed in triplicate to an agonistic anti-Fas antibody or a control antibody, at 200 ng/ml, in the presence or absence of i+ or i- RBCs, as indicated. After an additional 24 h, the cells were harvested and assessed for intact chromatin by the JAM assay. Each panel represents the results for cells of one initial culture circumstance, as indicated. Within each panel, the bars demonstrate the effects of reagents added at the 48 h time point.

FIGURE 4.

Model for inhibition of Fas-mediated apoptosis in HBL-3 cells by RBC antigen. In the context of a B lymphocyte binding antigen through IgM at the surface, as in an HBL-3 cell binding to “i” at the RBC surface, an activated CD4+ T cell recognizing the same antigen would provide “help” to that B cell by CD40L, expressed at the T cell surface. Upon CD40 engagement, the germinal center or malignant BL cell is induced to synthesize DNA, differentiate, and express activation antigens including Fas. The activated, Fas-expressing B cell is primed for apoptosis via FasL, which is also expressed at the surface of activated CD4+ T cells. When the Fas receptor is engaged by FasL, apoptosis is inhibited by cross-linking of the antigen receptor (IgM). In HBL-3 lymphoma cells, in which IgM binds “i” Fas-mediated apoptosis of the tumor cells is reduced in the presence of i+ RBCs, but not in the presence of I+ (i-) RBCs.