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*J Immunol* 2001; 167:5404-5411;
doi: 10.4049/jimmunol.167.9.5404
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Resistance to Fas-Mediated Apoptosis in EBV-Infected B Cell Lymphomas Is Due to Defects in the Proximal Fas Signaling Pathway

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Post-transplant lymphoproliferative disorder is characterized by the outgrowth of EBV-infected B cell lymphomas in immunosuppressed transplant recipients. Using a panel of EBV-infected spontaneous lymphoblastoid cell lines (SLCL) derived from post-transplant lymphoproliferative disorder patients, we assessed the sensitivity of such lymphomas to Fas-mediated cell death. Treatment with either an agonist anti-Fas mAb or Fas ligand-expressing cells identifies two subsets of SLCL based on their sensitivity or resistance to Fas-driven apoptosis. Fas resistance in these cells cannot be attributed to reduced Fas expression or to mutations in the Fas molecule itself. In addition, all SLCL are sensitive to staurosporine-induced cell death, indicating that there is no global defect in apoptosis. Although all SLCL express comparable levels of Fas signaling molecules including Fas-associated death domain protein, caspase 8, and caspase 3, Fas-resistant SLCL exhibit a block in Fas-signaling before caspase 3 activation. In two SLCL, this block results in impaired assembly of the death-inducing signaling complex, resulting in reduced caspase 8 activation. In a third Fas-resistant SLCL, caspase 3 activation is hindered despite intact death-inducing signaling complex formation and caspase 8 activation. Whereas multiple mechanisms exist by which tumor cells can evade Fas-mediated apoptosis, these studies suggest that the proximal Fas-signaling pathway is impeded in Fas-resistant post-transplant lymphoproliferative disorder-associated EBV+ B cell lymphomas. The Journal of Immunology, 2001, 167: 5404–5411.

Program in Immunology and Department of Surgery, Stanford University School of Medicine, Stanford, CA 94305

Received for publication May 14, 2001. Accepted for publication August 27, 2001.

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This work was supported by National Institutes of Health Grant R01AI41769 (to O.M.M.) and the Lucile Salter Packard Foundation. A.L.S. was supported by a Howard Hughes predoctoral fellowship in Immunology and a Stanford Graduate Fellowship. L.J.C. was supported by a fellowship from the Northern California Kidney Foundation.

A.L.S. and L.J.C. contributed equally to this work.

Address correspondence and reprint requests to Dr. Olivia M. Martinez, Stanford University School of Medicine, 1201 Welch Road, MSLS P312, Stanford, CA 94305-5492. E-mail address: omm@stanford.edu

Abbreviations used in this paper: FasL, Fas ligand; DISC, death-inducing signaling complex; DISC, death-inducing signaling complex; FLICE, FADD-like IL-1β-converting enzyme (FLICE) through homologous interactions between death effector domains on both molecules (4, 5), resulting in the autocatalytic cleavage of procaspase 8. The active caspase 8 heterotetramer is released from DISC and initiates the caspase cascade by cleaving the effector caspase 3. Effector caspase activation leads to DNA fragmentation and degradation of key cellular proteins, completing the cell death program.

Tumor cells have evolved multiple mechanisms to evade Fas-mediated apoptosis (6). Elevated levels of Bcl-2, a well-characterized anti-apoptotic molecule first discovered in follicular B cell lymphomas, are found in many different human cancers (7, 8). Expression of functional FasL, by the tumor cell itself may also confer protection by deleting anti-tumor lymphocytes, a phenomenon known as tumor counterattack (2). Additionally, alternative splicing of Fas transcripts can give rise to soluble forms of Fas, which have been proposed to contribute to apoptosis resistance by competing with membrane-bound Fas for available FasL molecules (9). Furthermore, the loss of function lpr and gld mutations in Fas and FasL, respectively, associated with autoimmune lymphoproliferative disease in mice in which activated lymphocytes are not eliminated by apoptosis also serve to demonstrate the potential for tumor development through Fas dysregulation (10, 11). More recently, viruses such as Kaposi’s sarcoma-associated human herpesvirus and human molluscipoxvirus were found to encode catalytically inactive homologues of caspase 8 known as FLICE inhibitory proteins that are suggested to act as dominant negative proteins that interfere with Fas-induced DISC formation (12–14). Indeed, malignancies linked to oncogenic viruses often demonstrate resistance to apoptosis, although the specific mechanisms through which viruses directly or indirectly prevent cell death programs within tumor cells remain elusive.

Post-transplant lymphoproliferative disorder (PTLD) is a significant complication in immunosuppressed patients following organ
transplantation (15, 16). PTLD is a phenotypically complex disorder ranging from benign hyperplasia to malignant lymphoma and is most commonly characterized by the development of EBV-associated B cell lymphomas (16, 17). EBV-specific CTL normally control the population of EBV-infected B cells in immunocompetent individuals, precluding tumorigenicity. However, immunosuppressive drugs used to prevent graft rejection deplete EBV-specific T cells and permit the hyperproliferation and lymphomagenesis of B cell hosts by EBV, a potent transforming virus in this context. EBV infection is of particular concern in pediatric transplant recipients, since primary infection of seronegative transplant patients is more likely to lead to PTLD (15). Currently, treatment of PTLD primarily involves the reduction of immunosuppressive therapy to restore the T cell compartment, with the unfortunate consequence of increasing the risk of graft rejection. Conversely, potent immunosuppression may select for highly proliferative B cell lymphomas that cannot be brought to regression later by competent EBV-specific CTL.

To study these B cell lymphomas in vitro, we previously generated EBV-infected spontaneous lymphoblastoid cell lines (SLCL) derived from transplant recipients diagnosed with PTLD. We demonstrated that IL-10 is an important autocrine growth factor for these lymphomas (18). To date, mechanisms of apoptosis in PTLD-derived B cell lymphomas have not been examined in detail. In this study we investigate programmed cell death triggered by Fas in these cells and demonstrate that two subsets of SLCL can be identified based on their sensitivity or resistance to Fas-mediated apoptosis. We show that resistance to Fas-driven apoptosis in SLCL is not due to alterations in the Fas molecule itself, but rather occurs via a block in Fas signal transduction that precludes caspase 3 activation. Thus, defects in the proximal Fas signaling pathway contribute to Fas resistance in PTLD-associated EBV B cell lymphomas.

**Materials and Methods**

**Cell lines and reagents**

SLCL are EBV-transformed B cell lines derived from the peripheral blood (MF4, VB5, JB7, JC62) or lymph node (AB5) of patients diagnosed with PTLD. As previously described, the SLCL are phenotypically, morphologically, and functionally similar to EBV B cell lymphomas that arise in vivo during PTLD (18). The AB5 cell line was generated from a lymph node tumor biopsy taken from a pediatric kidney recipient diagnosed with PTLD. The tumor mass was disrupted into a single-cell suspension, established and grown in complete medium (see below) without exogenous growth factors or cyclosporine A. AB5 was classified and grown as an SLCL according to published criteria as an SLCL accorded with 5% CO2. Mouse L° cells were maintained in DMEM with 10% heat-inactivated AB5 was classiﬁed and grown in complete medium (see below) without exogenous FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (BD Biosciences, Mountain View, CA). Gates were established on viable cells using forward and side scatter parameters, and apoptotic cells were quantiﬁed as the percentage of treated cells with increased EGFP ﬂuorescence compared with untreated cells (−IgM control Ab).

**Fas cell surface expression**

To determine Fas (CD95) expression, 1 × 10^6 cells were washed twice in cold HBBS containing 0.2% BSA and 0.02% sodium azide (FACS buffer) and incubated for 30 min on ice with 10 μg/ml PE-conjugated mAb to human Fas (BD PhaMingen, San Diego, CA) or an isotype-matched control Ab in 100 μl FACS buffer. After washing twice in FACS buffer, 20,000 cells were analyzed by flow cytometry. Only viable cells established using forward and side scatter parameters were used for analysis.

**Amplification and cloning of Fas gene transcripts**

Total RNA was isolated from the SLCL and Jurkat cells using TRIzol reagent (Life Technologies). Three micrograms of total RNA was used in cDNA synthesis reactions with oligo(dT) primers and AMV reverse transcriptase (Roche, Indianapolis, IN). Fas transcripts were ampliﬁed using the following PCR oligonucleotide primers: 5′-GAGTTATGTCCTCTAGGGGTTGGG (sense) and 5′-TATGTGGTCCTTCAGCGGCTA-3′ (antisense; Life Technologies), which span the full-length 1167-bp coding region of the Fas gene. PCR was performed using Gene Amp PCR core reagents (PerkinElmer, Branchburg, NJ). After an initial 5-min denaturation at 95°C, PCR amplification was conducted for 35 cycles of denaturation for 70 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 72°C. PCR products were cloned into the pED Adv vector (Clontech, Palo Alto, CA), and individual clones were sequenced by the Stanford University Proteomics and Nuclear Acid facility. Sequence analysis was performed using the DNAstar Lasergene for Windows software package (DNASTar, Madison, WI).

**Western blots**

For analysis of endogenous protein levels, 1 × 10^6 cells were washed in cold PBS and lysed in 100 μl 50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 5 μg/ml each of aprotinin and leupeptin, and 0.5 μg/ml pepstatin A on ice for 30 min. Lysates were centrifuged at 13,000 rpm for 5 min at 4°C to remove insoluble material, and the protein concentration of the lysates was determined using the DC protein assay (Bio-Rad, Hercules, CA). Thirty micrograms of each lysate was separated by 12% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Following blocking with 5% BSA in TBST, blots were incubated for 2 h with primary antibodies diluted in 3% BSA in TBST. The following primary Abs were used: rabbit anti-human caspase 3 (BD PhaMingen; diluted 1/1,000), and mouse anti-human caspase 2 (BD PhaMingen; diluted 1/1,000); rabbit anti-human caspase 3 (BD PhaMingen; diluted 1/1,000), and mouse anti-human β-actin (AC-15; diluted 1/8,000). Following three 5-min washes in TBST, bound Abs were detected using appropriate secondary Abs (peroxidase-conjugated goat anti-rabbit IgG; Jackson Immunoresearch Laboratories, West Grove, PA) or peroxidase-conjugated sheep anti-mouse Ig (Amersham Pharmacia, Piscataway, NJ; diluted 1/2,000) for a 1-h incubation at room temperature. Blots were washed as described above and developed using an ECL kit (Amersham Pharmacia).

To assess caspase activation following Fas ligation, 5 × 10^6 cells (1 × 10^6 cells/ml) were cultured for 0, 2, or 4 h at 37°C with 200 ng/ml mouse anti-Fas (CH-11), and lysates were prepared as described above. Western blotting was performed on 30 μg of each lysate as described above using rabbit anti-human caspase 8 or rabbit anti-human caspase 3 capable of

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To cite this article, use the DOI: 10.4049/jimmunol.1701897
detecting both pro and cleaved forms of caspase 8 and caspase 3, respectively.

**Immunoprecipitation of Fas-DISC complexes**

Immunoprecipitation of the Fas-DISC was performed as described previously (20). A total of 1 × 10^7 cells in 1 ml were cultured in the presence of 3 μg anti-Fas mAb APO1–3 IgG3 at 37°C for 2 h. Cells were washed once in cold PBS and lysed in 1 ml lysis buffer (30 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, 5 μg/ml each of aprotinin and leupeptin, and 0.5 μg/ml pepstatin A) for 15 min on ice. Following removal of insoluble material by centrifugation, Fas-DISC complexes were precipitated using 10 μl GammaBind Sepharose beads (Amersham Pharmacia) for 4 h at 4°C. Precipitates were washed four times in 1 ml cold lysis buffer and resuspended in 20 μl reducing Laemmli SDS-sample buffer. Samples were boiled for 3 min, separated by 12% SDS-PAGE, and transferred to nitrocellulose. Membranes were immunoblotted with anti-FADD and anti-caspase 8 Abs as described above or with anti-Fas (C20, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1/1000) Abs and detected by ECL.

**Caspase colorimetric assays**

Caspase 8 and caspase 3 activities were examined using commercially available colorimetric kits (BioVision, Palo Alto, CA). Briefly, 5 × 10^6 cells (1 × 10^6 cells/ml) were cultured for 24 h with anti-Fas mAb (CH-11) or an IgM isotype control Ab as described above. Cells were recovered, washed twice using PBS/10% FBS, and lysed with the provided buffer for 10 min on ice. Lysates were centrifuged at 13,000 rpm for 5 min at 4°C to remove insoluble material, and the protein concentration of the lysates was determined using the DC protein assay (Bio-Rad). The substrates Asp-Glu-Val-Asp-p-nitroaniline (pNA) or Ile-Glu-Thr-Asp-pNA (200 μM, final concentration) were incubated with 80 μg total protein, and the OD at 405 nm was determined at 1 and 120 min for both caspase 3 or caspase 8, respectively. The data are recorded as the OD405 at 120 min divided by the OD at 1 min.

**Results**

**Sensitivity of SLCL to Fas-mediated apoptosis**

To study EBV^+^ B cell lymphomas associated with PTLD, we generated several SLCL from the peripheral blood of pediatric transplant patients diagnosed with PTLD following liver transplantation. These B cell lines were transformed by EBV in vivo and were propagated from primary culture of PBMC in the absence of exogenous virus or growth factors. In one case (AB5) the SLCL was derived from a lymph node tumor biopsy originating from a kidney transplant patient with PTLD. We have previously shown that these cell lines possess an activated B cell phenotype and depend on IL-10 as an autocrine growth factor (18).

A panel of five SLCL was tested for sensitivity to Fas-induced apoptosis using the Fas cross-linking mAb CH-11. Following 24-h treatment with CH-11, cell cycle analysis with PI was used to distinguish apoptotic cells with sub-G1 hypodiploid DNA content. Annexin V staining was also used to identify cells in the early stages of apoptosis. Both PI analysis and annexin V staining identified two subsets of SLCL based on relative sensitivity to Fas-driven apoptosis, and the histograms of representative cell lines are shown in Fig. 1, A and B. Two SLCL, VB5 and MF4, showed similar Fas-dependent increases in apoptosis compared with the

**FIGURE 1.** Sensitivity of SLCL to Fas-mediated apoptosis. A, Cells (1 × 10^6 cells/ml) were cultured in 1 ml 200 ng/ml anti-Fas mAb CH-11 or an IgM isotype-matched control mAb. After 24 h cells were stained with PI and analyzed by flow cytometry. The percentage of viable cells with hypodiploid DNA content is indicated on the left of each histogram. Data from four representative cell lines are shown. B, Cells were cultured as described above, stained with annexin V-EGFP, and analyzed by flow cytometry. The percentage of annexin V-positive apoptotic cells is shown for each cell line. Data are the mean ± SEM of three separate experiments.
control Jurkat T cell line as demonstrated by both PI analysis and annexin V staining (Fig. 1, C and D). In contrast, the SLCL JC62, JB7, and AB5 all exhibited marked resistance to Fas-mediated apoptosis (Fig. 1, C and D). Daudi cells, an EBV+/Burkitt’s lymphoma line, served as an additional control for Fas resistance. A higher percentage of apoptotic cells was identified by annexin V staining compared with PI analysis, reflecting cells in the early stages of apoptosis before DNA fragmentation (Fig. 1, compare D with C). Nevertheless, the same pattern of relative sensitivity to Fas-induced apoptosis emerged when measured by either assay. To verify that the Fas-resistant SLCL were not simply refractory to CH-11 stimulation, SLCL were cultured with mouse L cell fibroblasts expressing recombinant FasL. The same pattern of Fas-induced apoptotic sensitivity and resistance by SLCL was seen (data not shown), demonstrating that the response by SLCL to Fas ligand is physiologically relevant.

Fas cell surface expression

To begin to address the differential sensitivity to Fas-mediated cell death in our cell lines, SLCL were stained for surface expression of Fas and analyzed by flow cytometry. All five SLCL express high levels of Fas compared with the Fas+ Jurkat T cells, whereas Daudi cells express low levels of Fas (Fig. 2). Although it should be noted that VB5 and MF4 cells have slightly higher levels of Fas expression than the other SLCL, resistance to Fas-induced apoptosis in JC62, JB7, and AB5 cells cannot be explained by a marked difference in the extent of Fas expression. Indeed, Jurkat T cells demonstrate high levels of Fas-mediated apoptosis (~70%; Fig. 1C) despite expressing less Fas than the Fas-resistant SLCL. Furthermore, cloning and sequencing of the Fas molecule from each SLCL revealed no sequence mutations or deletions (data not shown), implying the Fas receptor itself is not defective in Fas-resistant cell lines.

Staurosporine-induced apoptosis in SLCL

We next tested whether SLCL were sensitive to a non-Fas-dependent apoptotic stimulus using the protein kinase inhibitor staurosporine. All SLCL underwent increased apoptotic cell death following 24-h treatment with staurosporine as measured by cell cycle analysis (Fig. 3). The SLCL AB5 showed less cell death in response to staurosporine, which may reflect broader resistance to apoptotic stimuli in the cells that were derived from a more advanced, solid tumor. In addition, Daudi cells consistently demonstrated resistance to staurosporine-induced apoptosis, suggesting that Daudi cells have additional mechanisms to resist apoptotic stimuli. Nevertheless, these results indicate that none of the SLCL possesses a significant global defect in programmed cell death.

Expression of Fas signaling molecules in SLCL

Following our observations of differential sensitivity to Fas-induced apoptosis in the SLCL, we examined components of the Fas signaling pathway in these cell lines to explore the possible mechanism of Fas resistance. First, basal levels of important Fas signaling proteins in each cell line were determined by Western blotting of lysates prepared from untreated cells. The expression of FADD, caspase 8, and caspase 3 in all five SLCL examined was comparable to that in Fas-sensitive Jurkat control cells (Fig. 4), implying that each SLCL contains the critical proteins necessary for propagating signals for cell death following ligation of the Fas receptor.

DISC formation in SLCL

To determine whether there are defects in DISC formation in Fas-resistant cell lines, experiments were conducted to compare the recruitment of FADD and caspase 8 to the Fas-induced DISC in each SLCL. Following cross-linking of the Fas receptor with the agonist anti-Fas mAb APO1–3, immunoprecipitated protein complexes were examined by Western blotting to compare levels of coimmunoprecipitated FADD and caspase 8 in each cell line. Treatment with 1 μg/ml APO1–3 mAb induced a similar pattern of relative sensitivity to Fas-mediated apoptosis when assayed by cell cycle analysis (data not shown). As expected, both FADD and caspase 8 coimmunoprecipitated with the Fas receptor in the sensitive VB5 and MF4 cells following anti-Fas treatment, suggesting...
that DISC formation is intact in these cell lines (Fig. 5, lanes 14 and 16). Furthermore, bands corresponding to activated forms of caspase 8 (p43/41, p23) were clearly detected in VB5 and MF4 cells following 2 h of anti-Fas treatment, implying that the Fas-induced DISC in these cells is also functional for signaling. However, significantly reduced amounts of FADD and caspase 8 were found in the Fas-DISC complexes precipitated from JB7 and AB5 cells, consistent with their resistance to Fas-driven apoptosis (Fig. 5, lanes 10 and 12). This result suggests that a block or defect in Fas signaling exists at the level of DISC formation in these Fas-resistant cell lines. Interestingly, the small amounts of caspase 8 that are detected in JB7 and AB5 lysates also display some degree of activation, as demonstrated by the presence of p43/41 and p23 cleavage products.

Surprisingly, JC62 cells that are resistant to Fas-mediated apoptosis have higher levels of FADD and caspase 8 in the Fas-induced DISC than the resistant JB7 and AB5 cell lines (Fig. 5, compare lane 8 to lanes 10 and 12). This implies that a defect in Fas signaling may occur farther downstream in the JC62 cell line. Conversely, the p23 subunit of activated caspase 8 is much less prevalent in the JC62 Fas-DISC consistent with the lower levels detected in the other Fas-resistant cells. This reduction could contribute to the hampered Fas signaling in the JC62 cells despite proper DISC formation.

As an additional positive control for DISC formation, the EBV-transformed B cell line SKW 6.4 was used. Previous reports have established SKW 6.4 cells as type I cells, for which most pro-caspase 8 molecules are activated at the DISC following Fas ligation (21). Consistent with these findings, SKW 6.4 cells demonstrated significant DISC formation following anti-Fas treatment, with large amounts of caspase 8 and FADD coprecipitating with the Fas receptor (Fig. 5, lane 6), similar to the VB5 and MF4 SLCL.

Jurkat T cells demonstrate no appreciable DISC formation upon Fas ligation, as evidenced by the lack of detectable FADD or caspase 8 recruitment to the Fas receptor (Fig. 5, lane 2). This observation is consistent with previous studies establishing Jurkat cells as type II Fas-sensitive cells, in which almost all caspase 8 activation occurs without DISC formation (21) (Fig. 6). Daudi cells also show no detectable FADD or caspase 8 recruitment following Fas cross-linking, which may be partially explained by the lower levels of cell surface Fas expression observed by both flow cytometry (Fig. 2) and Western blotting (Fig. 5, lanes 3 and 4). It should be noted that less Fas receptor was detected in Jurkat and Daudi cells treated with anti-Fas to induce DISC formation before lysis (Fig. 5, lanes 2 and 4, +), as opposed to control samples in which the anti-Fas mAb was added after lysis (Fig. 5, lanes 1 and 3, −). It is likely that some anti-Fas mAb was lost while washing the cells that were treated with Ab before lysis, explaining why less Fas receptor was immunoprecipitated in those samples. Indeed, a faint band corresponding to the APO1–3 Ab H chain can be seen in all samples treated with Ab after lysis due to cross-reactivity with the peroxidase-conjugated secondary Abs, implying that more immunoprecipitating Ab is present in those precipitates (Fig. 5, − lanes). When supplementary Ab is added to lysates made from cells treated with APO1–3 before lysis, comparable amounts of Fas was immunoprecipitated (data not shown).

Caspase 8 and caspase 3 activation in SLCL

Results derived from examining DISC formation in each SLCL led us to investigate the kinetics of caspase 8 and caspase 3 activation following anti-Fas treatment. Cells were stimulated with the anti-Fas mAb CH-11 for 0, 2, or 4 h, and lysates made from these cells were immunoblotted with Abs capable of recognizing both thezymogen and cleaved forms of each caspase. In Jurkat T cells, both caspase 8 and caspase 3 activation peaked at 2 h, as evidenced from the presence of distinct bands corresponding to the p43/41 and p17 cleavage products of caspase 8 and caspase 3, respectively (Fig. 6, lane 2). By 4 h the levels of these activated subunits only decreased slightly (Fig. 6, lane 3). These results agree with previous reports on caspase 8 and 3 activation in Jurkat cells (21).

In Fas-sensitive VB5 and MF4 SLCL, both caspase 8 and caspase 3 are activated with similar kinetics compared with Jurkat T cells (Fig. 6, lanes 16–21), even though most caspase 8 activation is presumably occurring within the Fas-induced DISC in these

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**FIGURE 5.** DISC formation in SLCL following Fas ligation. The recruitment of FADD and caspase 8 to the Fas receptor was assessed in each cell line by cross-linking Fas with the anti-Fas mAb APO1–3 for 2 h (+) and immunoprecipitating Ab-bound complexes using protein G-Sepharose beads. As a control, APO1–3 was added to untreated cells after lysis (−). Coprecipitating proteins were detected by Western blotting using the Abs indicated on the left. The positions of the detected proteins are indicated on the right. IgH, H chain of the immunoprecipitating anti-Fas mAb. The blots shown are representative of at least three separate experiments.
cell lines as opposed to type II Jurkat cells. Interestingly, the presence of activated forms of both caspases decreases by 4 h of treatment in conjunction with an apparent increase in the amounts of zymogen forms (Fig. 6, lanes 18 and 21). This result is even more pronounced at 8 h (data not shown), suggesting that both caspase 8 and caspase 3 activations are transient in these cells despite efficient Fas-induced apoptosis by 24 h of treatment.

In Fas-resistant JB7 and AB5 cells, little or no caspase 8 and caspase 3 activation is detected by Western blot (Fig. 6, lanes 10–15), consistent with the levels of caspase activation in the DISC immunoprecipitation experiments (Fig. 5). The absence of cleaved subunits of either caspase persisted even after 8 h of Fas ligation (data not shown), suggesting the activation of caspase 8, and subsequently caspase 3, in these cell lines is not delayed, but rather is substantially blocked. In contrast, the activated p43/41 forms of caspase 8 are readily detectable in Fas-resistant JC62 cells (Fig. 6, upper panel, lanes 8 and 9), also consistent with the presence of these activated subunits in the DISC. However, the activated p17 form of caspase 3 is absent in JC62 cells following Fas cross-linking (Fig. 6, lower panel, lanes 8 and 9), suggesting a potential Fas signaling defect in this cell line that can be localized between caspase 8 and caspase 3 activation. Daudi cells again served as a negative control, showing no detectable caspase activation (Fig. 6, lanes 5 and 6) even after 8 h of anti-Fas treatment (data not shown). Caspase activation was also confirmed using colorimetric assays, in which specific pNA-labeled peptide substrates for caspase 8 and caspase 3 are incubated with cell lysates treated for 24 h with anti-Fas mAb or an IgM control. Substrate cleavage by activated caspases releases free pNA, which can be quantified at 405 nm on a spectrophotometer. Data generated from colorimetric assays confirmed the same pattern of caspase 8 and caspase 3 activation noted by Western blot for all cell lines (data not shown). Thus, the block in Fas-mediated apoptosis in resistant SLCL appears to occur before caspase 3 activation, probably resulting from inhibition of caspase 8 activation in the DISC or inhibition of caspase 3 cleavage by activated caspase 8.

Discussion

In this study we investigated Fas-mediated apoptosis in a panel of B cell lymphomas (SLCL) derived from transplant patients with PTLD, a severe complication arising from the uncontrolled outgrowth of EBV-infected B cells in the context of immunosuppression. We show that a subset of these SLCL is resistant to Fas-induced apoptosis when triggered by either an agonist anti-Fas mAb or FasL-expressing cells. This resistance is not due to reduced Fas expression at the cell surface or to mutations in the full-length Fas receptor that might render it incapable of oligomerization or signaling. Instead, we show that proximal Fas signaling is interrupted in resistant SLCL before appreciable caspase 3 activation. Defects or blocks in the Fas signaling pathway contribute to the impairment of proper DISC formation following Fas stimulation in two cell lines (JB7 and AB5), whereas in a third Fas-resistant cell line (JC62), caspase 3 activation is blocked despite intact DISC formation and caspase 8 cleavage. In contrast, the Fas signaling pathway is functional in Fas-sensitive SLCL (VB5 and MF4), driving the activation of the effector caspase 3 and leading to increased apoptosis.

Cells can be classified into two types according to the Fas signaling pathway used (21). In type I cells caspase 8 is primarily activated at the DISC, as seen in the SKW 6.4 B cell line and our Fas-sensitive SLCL. Caspase 8 activation is evident in the DISC as early as 10 min following Fas engagement (data not shown). In contrast, type II cells such as Jurkat T cells demonstrate reduced DISC assembly, with caspase 8 activation following loss of mitochondrial membrane potential. Although we have yet to extensively study the role of mitochondria in SLCL following Fas stimulation, our data suggest that Fas-resistant JB7 and AB5 cells are not type II cells. Despite impaired DISC formation, the low amounts of activated caspase 8 detected in our blotting experiments appear to be cleaved at the DISC level. Furthermore, we do not detect large amounts of activated caspase 8 following 2 h of anti-Fas treatment in these Fas-resistant SLCL, as would be expected for type II cells.

Fas signaling in JC62 cells appears to be obstructed before caspase 3 activation despite substantial caspase 8 activation at the DISC and the presence of undiminished levels of procaspase 3. It is difficult to discern whether this defect may be due to the specific targeting of caspase 3 or suboptimal generation of activated caspase 8 subunits (i.e., p23 subunit) for signal propagation. One novel explanation is that activated caspase 8 is not released from the DISC to cleave downstream substrates, although this is unprecedented in other Fas-resistant cell types. Another explanation might involve the inhibitor of apoptosis (IAP) family of proteins, which can specifically bind to and inhibit caspase 3 (22, 23). In fact, the human IAP survivin is often expressed in human neoplasms at higher levels than in normal adult tissues (24). IAPs could potentially inhibit caspase 3 activity and subsequent apoptosis in JC62 cells following Fas stimulation, while allowing staurosporine-induced apoptosis to occur through the release of Smac/DIABLO, an IAP inhibitor, from the mitochondria (25, 26).

In the Fas-resistant JB7 and AB5 cell lines, a defect in Fas signaling was detected at the level of DISC formation, resulting in suboptimal recruitment of both FADD and caspase 8 molecules. Although DISC formation appears to be impaired in these resistant...
cells, the caspase 8 molecules that are recruited to the DISC are capable of autocatalytic cleavage. However, the activated caspase 8 generated appears to be insufficient to induce the downstream apoptotic caspase cascade. Hence, the completion of the Fas-driven apoptotic program may require a higher threshold of caspase 8 activation than generated in JB7 and AB5 cells. Alternatively, the kinetics of DISC formation and/or caspase 8 activation may be delayed in these Fas-resistant cell lines, which may also be attributed to a specific block in proximal Fas signaling. The latter possibility is unlikely, however, since procaspase 8 remains uncleaved in JB7 and AB5 cells up to 8 h following Fas ligation (data not shown).

Another attractive possibility for explaining poor caspase 8 recruitment and activation at the Fas-induced DISC involves cellular FLICE inhibitory protein (cFLIP, also referred to as I-FLICE/FLAME-1/CASH/CLARP/Casper/MRT/Usurpin) (27–34). cFLIP exists as two isoforms, cFLIPL and cFLIPs, that structurally resemble caspase 8 and FLICE inhibitory protein, respectively (27, 32, 34). cFLIPL contains two death effector domains and a catalytically inactive caspase domain and has been proposed to have either an anti-apoptotic or a proapoptotic function in regulating Fas-mediated apoptosis depending on cellular context. For instance, Scalfid and colleagues (35) demonstrated that cFLIPL is uncleaved at the DISC upon Fas ligation in stably transfected JBAB cells and remains bound to prevent further recruitment of procaspase 8 into the DISC. Because this effect was only demonstrated when cFLIPL is overexpressed, endogenous levels of cFLIP may not play a significant role in blocking Fas signaling in SLCL. Furthermore, small amounts of procaspase 8 can be detected at the DISC following 2-h anti-Fas stimulation, suggesting that cFLIP is not blocking the continuous recruitment of procaspase 8. On the other hand, other groups have suggested the C-terminal caspase-like domain of cFLIPL is critical for inducing apoptosis in mammalian cells in overexpression studies (30–33). cFLIP is expressed in all SLCL at comparable levels (A. L. Snow, unpublished observations), and studies are ongoing to determine the role of cFLIP in Fas signaling in SLCL.

Research conducted on other types of B cell lymphomas to date reveals a myriad of mechanisms by which Fas-mediated apoptosis may be disrupted. For example, a recent report by Mueller and colleagues (36) described decreased Fas expression, activation of protein kinase C and NF-κB, and increased Fas expression all as distinct ways in which Fas-driven apoptosis is dysregulated in murine B cell lymphomas. Other groups have identified a soluble decoy receptor for Fas (DcR3) that is expressed in several types of tumors, including EBV-infected human B cell lymphomas (37, 38). Although this form of immune evasion could prove important for tumor protection against CTL and NK cells expressing FasL, it does not explain resistance to Fas-driven apoptosis in SLCL, even with an agonist anti-Fas Ab. More important, a recent report by Tepper and colleagues (39) uncovered defects in DISC formation and reduced caspase 8 activation in EBV-infected Burkitt’s lymphoma cells. They demonstrated that higher ratios of cFLIPL to caspase 8 transcripts in EBV+ Burkitt’s lymphoma cells correlate to increased resistance to Fas-mediated apoptosis, suggesting the modulation of this ratio may be a novel mechanism by which EBV imparts Fas resistance on Burkitt’s lymphoma hosts. Similar EBV-influenced mechanisms may also be at work in SLCL, which display a LCL phenotype as evidenced by constitutive expression of latent membrane protein-1 (LMP-1; A. L. Snow, unpublished observations).

LMP-1 itself has been implicated in host cell resistance to apoptosis through NF-κB-mediated up-regulation of Bcl-2 and related anti-apoptotic molecules (40–44). Latency in EBV-infected lymphoblastoid B cells is marked by high expression of LMP-1, which can engage TRAF molecules and force the constitutive activation of NF-κB in B cell hosts, including SLCL (A. L. Snow, unpublished observations). Both antisense targeting of LMP-1 and direct NF-κB inhibition can suppress growth and promote apoptosis in otherwise resistant EBV+ LCLs (45, 46). Although the up-regulation of Bcl-2 family members by LMP-1 is probably unrelated to direct type I Fas signaling, viral interference with death receptor-mediated apoptosis remains an intriguing possibility that has yet to be demonstrated in SLCL and related EBV+ cells.

Another study examining susceptibility to Fas-driven apoptosis in PTLD-derived cells showed that EBV+ LCL established from heart or bone marrow allograft recipients are extremely sensitive to apoptosis triggered by high dose agonistic anti-Fas Ab (47). Tumors arising from LCL inoculation in SCID mice could be forced into transient remission when treated with the anti-Fas Ab. In contrast, our study demonstrates that other PTLD-derived EBV+ B cell lymphomas can exhibit strong resistance to Fas-mediated apoptosis. Understanding this resistance on a molecular level, particularly in relation to viral modulation, may aid in the development of more effective therapeutic options involving Fas or other apoptotic stimuli for the treatment of PTLD-associated lymphomas.

Acknowledgments

We thank Dr. Maria Millan (Stanford University School of Medicine) for acquiring the AB5 lymph node biopsy and for helpful discussions.

References


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