Resistance of AIDS-associated Kaposi’s Sarcoma Cells to Fas-mediated Apoptosis

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ABSTRACT

Escape of tumor cells from apoptotic-mediated stimuli results in tumor cell survival and resistance to cytotoxic mechanisms. Kaposi’s sarcoma (KS) is the most common malignancy associated with AIDS, although its pathogenesis is not known. It is clinically important to determine whether AIDS-KS cells are resistant to apoptosis via the Fas system. Three isolates of AIDS-KS cells were studied. Although all KS cells express Fas on the cell surface, these cells were resistant to cytotoxic anti-Fas antibody (IgM, CH-11). Treatment of AIDS-KS cells with actinomycin D sensitized the tumor cells to anti-Fas cytotoxicity and apoptosis. Apoptosis was assessed by morphological changes and DNA fragmentation analysis. Three possible mechanisms related to AIDS-KS cells, resistance to anti-Fas cytotoxicity were examined. First, synthesis and secretion of soluble Fas by the tumor cells can neutralize antibody-induced cytotoxicity. However, none of the three types of KS cells expressed soluble Fas mRNA as determined by reverse transcription (RT)-PCR. Second, the expression of the proto-oncogene bcl-2 can protect cells from apoptotic signals. Analysis of bcl-2 mRNA by RT-PCR revealed that all three AIDS-KS cells express very low levels of bcl-2 mRNA. Third, the Fas-associated phosphatase-1 (FAP-1) is an antiapoptotic molecule reported to interact with Fas and can block transduction of the apoptotic signal. RT-PCR analysis revealed that all three types of AIDS-KS cells express high levels of FAP-1 mRNA, and treatment of KS cells with actinomycin D reduced the levels of FAP-1 mRNA significantly. These findings demonstrate that AIDS-KS cells are resistant to Fas-mediated apoptosis and suggest that FAP-1 may be involved in the acquisition of resistance of AIDS-KS to Fas anti-bodies-mediated apoptosis.

INTRODUCTION

KS was first described more than a century ago by Kaposi (1) as an indolent malignancy affecting elderly men of Ashkenazic Jewish and Mediterranean origin. Although KS was a rare disease until the 1980s, the number of patients with KS has increased with the prevalence of AIDS-KS. Now, it is estimated that approximately 30–50% of patients with HIV infection develop KS (2). KS causes edema, lymphatic obstruction, pulmonary edema, and bleeding from the KS itself and about 30% of AIDS-KS patients die from such complications (2). KS is also induced in recipients of organ transplantation due to long-term administration of immunosuppressive drugs (2). Spontaneous regression of KS has been observed in some patients with moderate immune deficiency (3). These clinical observations suggest that immune deficiency is an important factor in the pathogenesis of AIDS-KS.

Cytotoxic T lymphocytes or NK cells play a central role in immune surveillance. Recently, the Fas system has been invoked in the mechanism of target cell lysis by these killer cells (4, 5). Namely, the Fas ligand on the killer cells recognizes the Fas antigen receptor (Fas) on the target cells and triggers a signal of cell death or apoptosis (6). Fas is a receptor protein that transduces an apoptotic signal into susceptible cells and belongs to the tumor necrosis factor/nerve growth factor receptor superfamily (7, 8). Extensive studies indicate that induction of cell death via Fas, the so-called “Fas-mediated cytotoxicity,” is important in CTL-mediated cytotoxicity (4, 6) and development and homeostasis (6). In vivo studies show that mice with a defect of Fas (lpr mice) or Fas ligand (gld mice) develop autoimmune disease (6). Furthermore, tumorogenesis may be attributed, at least in part, to a disorder of the Fas system by down-modulation of signaling through the Fas. As a result of such modulation, tumor cells may escape from cell death and survive. Several mechanisms for modulation of Fas-mediated cytotoxicity have been reported, namely: (a) overexpression of bcl-2 (9); (b) production of soluble Fas (sFas) in which cells escape from cell death by neutralization of the Fas ligand (10); (c) overexpression of the FAP-1 (11) protein that presumably interacts with a “suppressive domain” of Fas; (d) insufficient expression of molecules that interact with the “death domain” of Fas, which is essential to transduce the apoptotic signal (12); and (e) mutation of the primary structure of Fas, thus rendering it inactive (6, 13).

This study examined whether AIDS-KS cells can undergo apoptosis through signaling of the Fas system. Nakamura et al. (14) established a long-term culture system of AIDS-KS cells, and the cells were maintained in culture by the addition of supernatants derived from retrovirus-infected T cells. Three isolates of AIDS-KS cells were used in this study, and the following questions were investigated: (a) whether AIDS-KS cells express Fas on the cell surface and whether they are sensitive to anti-Fas cytotoxic antibody; (b) whether protein synthesis inhibitors sensitize the cells to anti-Fas cytotoxicity and apoptosis; (c) whether AIDS-KS cells are protected from apoptosis by secretion of neutralizing sFas; and (d) whether AIDS-KS cells overexpress antiapoptotic genes like bcl-2 and FAP-1.

MATERIALS AND METHODS

Cells and Reagents. AIDS-KS cell cultures were established from the pleural effusion of lung KS (AIDS-KS-22), lung KS (AIDS-KS-3), and oral mucosa KS (AIDS-KS-10B) of HIV-1-infected patients. AIDS-KS-22 cells were established at the Institute of Molecular Medicine, Huntington Memorial Hospital (Pasadena, CA) as described previously (14). AIDS-KS-3 and AIDS-KS-10B were developed in the Laboratory of Tumor Cell Biology, National Cancer Institutes, NIH (Bethesda, MD: Ref. 14). AIDS-KS cells were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 15% fetal bovine serum and conditioned medium containing human oncostatin (OM)-expressing Chinese hamster ovary cells at a final concentration of 15 ng/ml OM. OM-expressing Chinese hamster ovary cells were established as described previously (15). Raji (CCL86) and HT-29 (HTB88) were purchased from American Type Culture Collection, and CEM was maintained in our laboratory. Anti-Fas IgM antibody (CH-11) and anti-Fas IgG antibody (UB2) were purchased from Kamiya Biochemical, Inc. (Thousand Oaks, CA). Antimouse IgG monoclonal antibody and phycoerythrin-conjugated IgG were purchased from AMAC, Inc. (Westbrook, ME) and BIOMEDA, Inc. (Forest City, CA), respectively. ActD, crystal violet, PI, and RNase A were purchased from Sigma (St. Louis, MO).

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3. The abbreviations used are: KS, Kaposi’s sarcoma; AIDS-KS, AIDS-associated KS; FAP, Fas-associated phosphatase-1; ActD, actinomycin D; sFas, soluble form of the Fas antigen receptor; NK, natural killer; OM, oncostatin M; RT, reverse transcription; PI, propidium iodide; XTT, sodium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); 3-[(4-methoxy-6-nitrophenyl)amine]-5-[(3-sulfophenyl)carboxamido]-2-(4-sulfophenyl)-2H-benzo[d]-1,3-oxidine-1-sulfonic acid hydrate.
Flow Cytometry. AIDS-KS cells were washed twice with PBS containing 1% BSA and 0.01% sodium azide. One μg UB2 was added to 2 x 10^5 cells in 50 μl cold PBS-BSA and incubated on ice for 60 min. The cells were washed twice in cold PBS-BSA and brought up in 1:100 dilution of phycoerythrin-conjugated antibody. After incubation for 60 min, the stained cells were subjected to flow cytometric analysis using an Epics (Coulter Electronics, Hialeah, FL) flow cytometer.

Cytotoxicity Assays. AIDS-KS cells (1 x 10^5 cells/0.5 ml/well) were seeded in a 24-well culture plate and cultured for 18 h in a 37°C incubator. Each well was washed with medium twice and incubated in medium in the presence of various concentrations of CH-11. Eighteen h later, the cells were fixed with 0.025% of glutaraldehyde and stained with crystal violet (0.2% in PBS). The dye was extracted with ethanol-0.1 m NaH_2PO_4 (1:1). Absorbance was read at 570 nm, and the percentage of cytotoxicity was calculated based on the standard curve of KS cell numbers. Cytotoxicity for CEM was determined using the cell proliferation kit-XTT assay (Boehringer Mannheim, Indianapolis, IN) as described in the instruction manual. Statistical analysis was performed using the student t test.

DNA Staining. Cells (1 x 10^5) were washed twice with PBS-BSA and incubated in 70% ethanol on ice. After 30 min of incubation, the cells were washed twice with PBS-BSA, and then 70 μl RNase A (1 mg/ml) and 140 μl PI (100 μg/ml) were added to each sample. After 1 h of incubation in the dark, DNA analysis was determined using an Epic C flow cytometer.

RT-PCR. Total RNA from AIDS-KS-3, KS-10B, and KS-22 cells and Raji, HT-29, and CEM cells were prepared using the guanidine-CsCl method. Total RNA from the liver and kidney were purchased from Clontech (Palo Alto, CA). The first-strand cDNA was synthesized in 20 μl reaction mixture from 2 μg total RNA using a cDNA preamplification kit (GIBCO-BRL). The PCR reaction (100 μl) was performed in a reaction mixture from cDNA synthesis, under the following conditions: 1 cycle each at 94°C for 3 min, 60°C for 2 min, and 72°C for 3 min; 28 cycles each at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min; and 1 cycle each at 94°C for 1 min, 60°C for 2 min, and 72°C for 7 min. Ten μl of the PCR reaction was subjected to analysis with agarose gel electrophoresis followed by ethidium bromide staining. The PCR was performed using the following upstream (U) and downstream (D) primers. Fas (U): 5'-GACCCAGAATACCAAGTGAGGTCTGCAGAGAAGCAAGAATAC-3' (11, 19); bcl-2 (U): 5'-CGACTTCGCCGAGATGTCAGATGTA-3'; Fas (D): 5'-CAGGmCAGGAmAAGGUGGAGA1T-3' (20); f3-actin(U): 5'-ATCGGACCACACCITCTACAATGCTCAGATAGGCACCCAG-3'; bcl-2 (D): 5'-AClTGTGAGGTCTGCAGAGAAGCAAGAATAC-3' (Clontech).

RESULTS

Expression of Fas on AIDS-KS Cells and Sensitivity to Anti-Fas Antibody-mediated Cytotoxicity

Three isolates of AIDS-KS cells (AIDS-KS-3, AIDS-KS-10B, and AIDS-KS-22) derived from different tissues of HIV-infected patients were established in culture. The KS cells were tested for surface expression of Fas by flow cytometry. All three KS cell isolates express significant levels of Fas on their cell surface (Fig. 1). The sensitivity of these KS cells to cytotoxicity by anti-Fas IgM antibody (CH-11) was examined by staining of viable cells with crystal violet.

The viability of KS-3 following 18-h treatment with different concentrations of CH-11 (up to 1000 ng/ml) was the same (>95%; Fig. 2A), whereas the control cell line CEM was completely killed at a concentration of 30 ng/ml (Fig. 2D). The viability of KS-10B (Fig. 2B) and KS-22 cells (Fig. 2C) was 80% and 55%, respectively, following treatment with CH-11 (1000 ng/ml). Since the growth of the KS cells is dependent on OM, it was possible that OM protected the KS cells from anti-Fas antibody-mediated cytotoxicity. However, similar results were obtained both in the presence or absence of OM (data not shown). These findings demonstrate that AIDS-KS cells are relatively resistant to anti-Fas antibody-mediated cytotoxicity, even though the KS cells express high levels of Fas on their cell surface.

Previously studies demonstrated that protein synthesis inhibitors like ActD and cyclohexamide sensitize some cells to anti-Fas antibody-mediated cytotoxicity. KS-22 cells were completely killed following treatment with 100 ng/ml CH-11 and 10 ng/ml ActD (Fig. 2C). Both KS-3 (Fig. 2A) and KS-10B (Fig. 2B) were also sensitized by ActD but not completely because 30–50% of the cells were still viable at high concentrations of CH-11. Treatment with ActD did not modify Fas expression (Table 1). These findings demonstrate that ActD sensitizes KS cells to anti-Fas antibody-mediated cytotoxicity.

Cell Death via Apoptosis

Previous findings indicated that cytotoxicity by anti-Fas antibody is mediated through programmed cell death and apoptosis. Cell death of ActD-treated KS cells by anti-Fas (CH-11) was examined for morphological changes and for DNA fragmentation by staining with PI. Following treatment of KS-10B with CH-11 (300 ng/ml) and ActD (10 ng/ml), the cells detached from the plate while treatment with either agent alone did not detach the cells. Phase-contrast microscopy revealed extensive blebbing as shown in Fig. 3A. Fluorescence-activated cell sorting analysis revealed DNA fragmentation of AIDS-KS cells (Fig. 3B), and the number of cells in the G_0–G1 decreased from 84% to 48% after treatment with ActD and CH-11. These findings demonstrate that killing of KS cells with ActD and anti-Fas antibody is mediated through programmed cell death or apoptosis.

Mechanism of KS Cells’ Resistance to Cytotoxic Anti-Fas Antibody

AIDS-KS Cells Express Fas mRNA but not sFas-mRNA. It has been reported recently that secretion of sFas can neutralize anti-Fas antibody-mediated cytotoxicity (10); sFas, therefore, could be responsible for AIDS-KS cells resistance to anti-Fas antibody-mediated cytotoxicity. The presence of sFas was examined by RT-PCR using specific primers that distinguish sFas mRNA from Fas mRNA. Clearly, all three AIDS-KS cells express Fas mRNA but not sFas mRNA (Fig. 4). Similar findings were found for the anti-Fas antibody-sensitive CEM cell line. These findings demonstrate that resistance of AIDS-KS cells to cytotoxic anti-Fas antibody is not due to secretion of sFas.
AIDS-KS Cells Express Low Levels of bcl-2. The expression of the antiapoptotic oncogene bcl-2 can inhibit apoptosis from many stimuli including anti-Fas antibody (9). Therefore, it was possible that bcl-2 was up-regulated in KS cells, thus rendering them resistant to anti-Fas antibody. The expression of bcl-2 mRNA in AIDS-KS cells was examined using RT-PCR, and Raji tumor cells were used as positive controls because they express large amounts of bcl-2 mRNA. All AIDS-KS cells expressed low levels of bcl-2 mRNA (Fig. 5). Furthermore, Western blot analysis for bcl-2 showed that the level of bcl-2 protein was very low (data not shown). These findings suggest that bcl-2 does not contribute to the protection of KS cells from cytotoxicity by anti-Fas antibody.

AIDS-KS Cells Express High Levels of FAP-1 mRNA. The expression of FAP-1 protects cells from apoptosis as reported in FAP-1 transfectants (11). All three types of AIDS-KS cells express high levels of FAP-1 mRNA, whereas FAP-1 mRNA expression was low in the anti-Fas-sensitive cell lines HT-29 and CEM (Fig. 6). The high level of FAP-1 mRNA in AIDS-KS cells was comparable to the level of FAP-1 mRNA in the kidney, an organ which expresses the highest levels reported for FAP-1 mRNA (11). The lack of FAP-1 mRNA in Raji and liver cells is consistent with other reports. Treatment of AIDS-KS with ActD reduced the levels of FAP-1 mRNA significantly (Fig. 6). However, the levels of β-actin mRNA, bcl-2 mRNA, and Fas mRNA were not affected by ActD (data not shown). These data suggest that FAP-1 may be one of the factors responsible for the resistance of KS cells to cytotoxic anti-Fas antibody.

**DISCUSSION**

Evidence is presented which demonstrates that AIDS-KS cells express Fas on the cell surface even though the cells are resistant to anti-Fas antibody-mediated cytotoxicity and apoptosis. However, treatment with ActD sensitizes the AIDS-KS cells to cytotoxicity by the anti-Fas antibody. Two potential mechanisms of AIDS-KS resistance to anti-Fas were not invoked, namely, secretion of neutralizing sFas and up-regulation of the antiapoptotic proto-oncogene bcl-2. However, there was up-regulation of FAP-1 in all three KS cultures, and FAP-1 has been shown to suppress Fas-mediated signaling (11). Treatment with ActD down-regulated significantly FAP-1 mRNA. These findings suggest that FAP-1 up-regulation correlates with the resistance of AIDS-KS cells to apoptosis by anti-Fas antibody.

**Table 1. Effect of ActD on Fas expression on AIDS-KS cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Background</th>
<th>No ActD</th>
<th>ActD (10 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS-3</td>
<td>23.4</td>
<td>72.9</td>
<td>76.9</td>
</tr>
<tr>
<td>(117)</td>
<td>(129)</td>
<td>(134)</td>
<td></td>
</tr>
<tr>
<td>KS-10B</td>
<td>28.8</td>
<td>63.1</td>
<td>69.1</td>
</tr>
<tr>
<td>(132)</td>
<td>(140)</td>
<td>(142)</td>
<td></td>
</tr>
<tr>
<td>KS-22</td>
<td>18.5</td>
<td>50.8</td>
<td>66.1</td>
</tr>
<tr>
<td>(123)</td>
<td>(131)</td>
<td>(136)</td>
<td></td>
</tr>
</tbody>
</table>

* AIDS-KS cells were cultured in the absence or presence of ActD (10 ng/ml) for 24 h and Fas expression on their cell surface [UB-2 (anti-Fas Ab-IgG)] was analyzed using flow cytometry. The numbers in parentheses represent mean fluorescence.
with autoimmune disease (10). The sFas is also expressed in some types of leukemia, and it has been suggested that sFas protects malignant cells from apoptosis mediated by the host immune surveillance (17). This study demonstrated that AIDS-KS cells express Fas mRNA but not sFas mRNA, thereby indicating that sFas does not function as an antiapoptotic protein in AIDS-KS cells. The protooncogene bcl-2 was identified in human follicular lymphomas (20). In both transfection experiments and transgenic mice, bcl-2 was shown...
to play a crucial role in the survival of hematopoietic and lymphoid cells (9). For instance, overexpression of bcl-2 in hematolymphoid cells blocks apoptosis induced by withdrawal of the growth factors, by addition of steroids, and by cytotoxic anti-Fas antibody (9). In addition, bcl-2 functions as an antiapoptotic protein in other types of cells, such as neurons and some malignant cells (9). In AIDS-KS cells, the level of bcl-2 expression is low when compared with other cells like Raji cells. Therefore, it seems unlikely that bcl-2 is a responsible factor for resistance of AIDS-KS cells to Fas-mediated cytotoxicity.

Deletion mapping studies on Fas demonstrated that the carboxyl-terminal 15-amino acid polypeptide is a "suppressive domain" that is involved in inhibition of the apoptotic signal (21). Recently, Sato et al. (11) identified a gene product FAP-1 which interacts with this suppressive domain. Transfection of FAP-1 cDNA into a T-cell line partially abolished Fas-mediated apoptosis. Furthermore, Sato et al. (11) showed that FAP-1 expression is highest in tissues and cell lines that are relatively resistant to Fas-mediated cytotoxicity. Likewise, the anti-Fas-resistant AIDS-KS cells express high levels of FAP-1 mRNA while two anti-Fas-sensitive cell lines, CEM and HT-29, express low levels of FAP-1 mRNA. The role of FAP-1 as a resistance factor was corroborated in experiments demonstrating a positive correlation between ActD sensitization of AIDS-KS cells to anti-Fas cytotoxicity and down-regulation of FAP-1 mRNA. FAP-1 may not be the only resistant factor since ActD-treated KS-3 cells were partially resistant to high concentrations of anti-Fas antibody. Thus, these studies suggest that FAP-1 contributes to KS cells for resistance to Fas-mediated cytotoxicity, but other molecules are also involved. For instance, the role of the non-FAP-1 protective protein has been reported in liver cells in primary cultures resistant to anti-Fas antibody, but ActD sensitizes the cells to anti-Fas antibody (16). However, FAP-1 mRNA was below detection in liver cells (Fig. 6).

Yonehara et al. demonstrated that Fas-mediated cytotoxicity in some cells was enhanced by treatment with IFN-γ. Weller et al. (22) also reported that the degree of Fas expression on the cell surface is the most important factor for cells’ sensitivity to anti-Fas antibody, and Fas expression is augmented by cytokines, like IFN-γ and tumor necrosis factor-α (22). Dysregulation of cytokine production in HIV-infected mononuclear cells or AIDS-KS cells is one of the pathological features of HIV infection (2). Hence, we examined whether IFN-γ
treatment may enhance AIDS-KS cells sensitivity to anti-Fas antibody. However, AIDS-KS cells were still resistant to anti-Fas antibody, although IFN-γ enhanced the expression of the MHC class II antigen on AIDS-KS cells (data not shown).

Resistance of AIDS-KS cells to Fas-mediated cytotoxicity may play an important factor in the pathogenesis of AIDS-KS. Reiter et al. (23) reported that AIDS-KS cells are resistant to NK cell-mediated cytotoxicity, although the underlying mechanism of resistance is not known. Based on the findings here, it is possible that NK cell resistance is attributable to resistance to Fas signaling since NK cells can lyse target cells by a Fas-dependent system (5). Aside from the dysfunction of immunocompetent cells in AIDS patients, the acquisition of resistance to Fas-mediated cytotoxicity by AIDS-KS cells may result in the survival of AIDS-KS cells and progression of AIDS-KS. Additional studies examining the sensitization of AIDS-KS cells to Fas-mediated cytotoxicity in vivo should provide a new therapeutic approach in the treatment of AIDS-KS.

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