Expression of Fas/Apo-1 (CD95) and Apoptosis in Tumor Cells From Patients With Plasma Cell Disorders

By Hiroyuki Hata, Hiromitsu Matsuzaki, Motohiro Takeya, Minoru Yoshida, Takashi Sonoki, Akitoshi Nagasaki, Noriomi Kuribayashi, Fumio Kawano, and Kiyoshi Takatsuki

Fas/Apo-1 antigen (CD95) is a cell surface molecule that directly mediates apoptosis. Fas expression was studied in five plasma cell lines, 11 multiple myeloma cases, and three plasma cell leukemia (PCL) cases. Induction of apoptosis by anti-Fas antibody was studied in five plasma cell lines and fresh plasma cells from eight patients. Apoptosis was confirmed by morphologic analysis alone or in combination with DNA electrophoresis analysis. Four of the five cell lines showed Fas expression, three of which showed induction of apoptosis by anti-Fas antibody. One cell line, RPMI 8226, showed the highest sensitivity for Fas-mediated apoptosis. High bcl-2 expression was found in KMS12PE, which showed resistance to Fas-mediated apoptosis despite its Fas expression. Plasma cells from seven fresh cases, including all five cases with high serum lactate dehydrogenase (LDH), showed expression of Fas antigen. Fas-induced apoptosis was found in five cases at various levels, although significant induction of apoptosis was found in only one case. Interestingly, Fas-independent apoptosis was induced during culture without anti-Fas antibody in cases with high serum LDH. These results indicate that plasma cells from aggressive myeloma with high LDH express Fas antigen and undergo apoptosis through either Fas-mediated or Fas-independent pathways. An understanding of the mechanism of apoptosis in malignant plasma cells should contribute to investigations of the pathophysiology of and therapy for myeloma/PCL.

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MATERIALS AND METHODS

Cell lines and fresh cells. The cell line RPMI 8226 was purchased from Dainippon Pharmaceutical Co Ltd (Tokyo, Japan). KHM1B10 and KHM111 were established from pleural effusion of aggressive myeloma with high LDH in our laboratory. KMS12BM and KMS12PE, which were established from the bone marrow and the pleural effusion, respectively, of the same myeloma patient,12 were gifts from Dr T. Ohnishi (Kawasaki Medical School, Kurashiki, Japan). KHM2B, which was used as a positive control for bcl-2, was established in our laboratory from a patient with Burkitt's lymphoma with t(14;18), resulting in high bcl-2 expression.13 Fresh cells were obtained from patients and separated by Ficoll Conray density gradient centrifugation (Sigma, St Louis, MO). Populations of plasma cells were assessed by morphologic analysis with May-Giemsa staining. In some experiments, viable cells were obtained from cryopreserved cells and separated by Ficoll Conray density gradient centrifugation.

Antibodies and flow cytometry. Very late antigen (VLA)4 was purchased from Cosmo Bio (Tokyo, Japan), CD45 was purchased from Becton Dickinson (Mountain View, CA), VLA5 was a gift from Dr K. Miyake (Saga Medical School, Saga, Japan), and anti-Fas antibodies CHI 1 (mouse IgM) and UB2 (mouse IgG1) were purchased from MBL (Nagoya, Japan). As CHI 1 is capable of inducing apoptosis and UB2 is not, these antibodies were used to induce apoptosis and to analyze expression of Fas antigen, respectively. Mouse IgM (MOPC 104E) was used as a negative control for CHI 1.

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Mouse IgG1 (Becton Dickinson) was used as a negative control in flow cytometry. Anti-mouse IgG (Organon Teknika Corp, West Chester, PA) was used as a second antibody in flow cytometry analysis. Flow cytometry analysis was performed by FACSscan (Becton Dickinson).

Western blotting. Antibodies to bcl-2 and p53 were purchased from Cambridge Research Biochemicals (London, UK) and Oncogene Science (Uniondale, NY), respectively, and used according to the manufacturers' directions. Molecular weight marker was purchased from GIBCO BRL (Grand Island, NY). Eighty micrograms of cell lysates was applied on a 10% acrylamide gel, electrophoresed, and transferred to nitrocellulose membrane. After staining with monoclonal antibodies, the membrane was stained by alkaline phosphatase-conjugated antimouse IgG (Promega, Madison, WI) and alkaline phosphatase substrate (Promega).

Electron microscopy. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer and postfixed with 1% osmium tetroxide in the same buffer. The specimens were then dehydrated and embedded in epoxy resin. Ultrathin sections, stained with uranyl acetate and lead nitrate, were observed with a Hitachi 12A electron microscope (Tokyo, Japan).

Cytokine. Recombinant interleukin-6 (IL-6) was obtained from Chugai Pharmaceuticals Co, Ltd (Tokyo, Japan). IL-6 at a concentration of 1 ng/mL was used in the experiments, because this concentration saturated the growth response of the IL-6-dependent cell line, 29360.11

Analysis of apoptosis. Cells or fresh cells were incubated in RPMI 1640 medium containing 10% fetal calf serum (FCS) at a cell concentration of 1 × 10^6/mL, for 2 hours at 37°C in humidified air containing 5% CO2 with or without 100 ng/mL of anti-Fas (CH11) antibody. In control experiments, purified MOPC 104E (mouse IgM) in RPMI 1640 medium containing 10% fetal calf serum (FCS) at a cell concentration of 1 × 10^6/mL, for 2 hours at 37°C in humidified air containing 5% CO2 with or without 100 ng/mL of anti-Fas (CH11) antibody was added at a concentration of 100 ng/mL. After incubation, 1 × 10^6 cells were applied to cytopsin slides and stained by May-Giemsa solution for morphologic analysis. Cells showing morphologic features typical of apoptosis, such as DNA fragmentation; a condensed nucleus, and relatively preserved cytoplasm, were judged as undergoing apoptosis. Apoptosis was determined by analyzing more than 100 cells microscopically. The ratio of cells undergoing apoptosis was calculated as percent apoptosis.

DNase was isolated from the remaining cells by an improved method to extract fragmented DNA according to Faschini et al. Briefly, pellets of approximately 1 × 10^6 cells were lysed in 500 μL of phosphate-buffered saline (PBS) containing 0.2% Tween 20 for 5 minutes at room temperature. Then cells were centrifuged at 15,000 rpm for 20 seconds to remove nuclei. Protein was removed from the recovered supernatant by treating with the same volume of phenol and chloroform. Nucleic acid in the supernatant was precipitated with 2 vol ethanol, 10 mmol/L magnesium chloride, and 0.3 mol/L sodium acetate, centrifuged, and resuspended in Tris-EDTA (TE) solution. After treating samples with 100 μg/mL of RNase A at 37°C for 10 minutes, DNA was precipitated in 2 vol of ethanol and 0.3 mol/L of sodium acetate, centrifuged, and suspended in 10 μL of TE. Extracted DNA solution (5 μL) was applied to a 2% agarose gel, electrophoresed, stained with 0.5 μg/mL of ethidium bromide, and photographed under ultraviolet (UV) illumination. Preliminary experiments in combination with morphologic analysis revealed that DNA extracted from as few as 1 × 10^4 apoptotic cells was required to visualize ladder formation. In some experiments, viable cell numbers were counted by dye exclusion assay after culturing cells with or without anti-Fas antibody for 24 hours.

RESULTS

**Fas expression and Fas-mediated apoptosis of plasma cell lines.** Fas expression was found in four of five plasma cell lines (Table 1). Although KMS12BM and KMS12PE were derived from the same patient and shared the same Ig gene rearrangement (data not shown), Fas was found on KMS12PE but not on KMS12BM. RPMI 8226 showed the highest level of expression of Fas. Significant apoptosis was induced by anti-Fas antibody (CH11) in a plasma cell line, RPMI 8226, which was confirmed by morphologic examination of May-Giemsa-stained samples, and electron microscopy (data not shown). Anti-Fas–treated RPMI 8226 cells showed DNA ladder formation after 2 hours in culture, which was not inhibited by IL-6 at a concentration of 1 ng/mL (data not shown). Percent apoptosis of cell lines that were treated with CH11 was correlated with the median fluorescence intensity of the staining with anti-Fas antibody (Table 1). Although other Fas-positive cell lines (KHM1B, KHM11, and KMS12PE) showed apoptosis at lower frequencies as observed by morphologic analysis of May-Giemsa–stained samples, DNA ladder formation was not found in these lines (data not shown) because of the low sensitivity of gel analysis. All cell lines showed no morphologic change after 2 hours’ incubation with control antibody. After 24 hours in culture with CH11, a significant increase of dead cells at more than 90%, was observed in RPMI 8226 (Fig 1). Induction of dead cells by anti-Fas antibody was also found in KHM1B, KHM11, and KMS12PE, although it was less than that found in RPMI 8226. Expression of bcl-2 and p53 in cell lines was analyzed by Western blotting (Fig 2). Expression of p53 was at almost the same level in all plasma cell lines. Low expression of bcl-2 was found in RPMI 8226, while no expression of bcl-2 was found in KHM1B, KHM11, and KMS12BM. The highest expression of bcl-2 was observed in KMS12PE, indicating inhibitory mechanism of bcl-2 for Fas-mediated apoptosis.

**Fas expression of fresh samples.** Fas expression of fresh cells from 14 clinical cases of plasma cell-related diseases was analyzed (Table 2). Cases with more than 10% expression of Fas were judged as positive. Four and 10 cases were Fas-negative and -positive, respectively. All cases of PCL (cases 12, 13, and 14) were Fas-positive. Although cases 3 and 4 had large tumor cell populations, they did not express Fas antigen. There was no correlation between Fas expression and source of plasma cells. The correlation between Fas and LDH, shown in Table 2, is analyzed in Fig 3. All of the patients with high LDH (cases 7, 9, 10, 13, and 14) showed expression of Fas, whereas all Fas-negative patients had normal LDH levels. Expression of VLA4 and VLA5 on fresh cells was studied to evaluate the differentiation stage of plasma cell lines.
plasma cells in 12 cases (Table 2). All fresh cases were positive for only VLA4, except for case 9, which was positive for VLA4 and CD45 (data not shown). On the other hand, a correlation between VLA4 and Fas expression was suggested (Fig 4). VLA4 expression was relatively lower in cases with low expression of Fas and higher in other cases with high expression of Fas. Statistical analysis suggested a correlation between VLA4 and Fas expression (r = .851). To avoid overestimation of Fas and VLA4 due to the contamination of lymphocytes, correlation between Fas and VLA4 expression was evaluated in samples containing more than 80% of tumor cells (cases 3, 4, 7, and 9 through 14). The correlation in expression of these two antigens was statistically significant (P = .04, analyzed by Spearman’s correlation test).

Fas-mediated apoptosis of fresh samples. Induction of apoptosis either by culture alone or with addition of anti-Fas antibody (CH11) was analyzed by morphologic analysis in eight cases (cases 2, 7, and 9 through 14) that provided large enough numbers of cells for further analysis. The percent apoptosis ratio, which was analyzed morphologically in fresh samples is shown in Fig 5. Cases 9 and 10 and all PCL cases (cases 12 through 14) showed Fas-induced apoptosis at various levels. DNA extraction was successful in only three of these cases (cases 10, 13, and 14) because of insufficient cell numbers from other cases. Significant Fas-induced apoptosis was confirmed by DNA ladder formation in case 10, although a small amount of DNA ladder was found at the control condition (Fig 6). Case 14 had less induction of apoptosis by anti-Fas antibody than did case 10, while tumor cells from case 13, which showed high induction of apoptosis even at the control condition, did not show increased DNA ladder formation by anti-Fas antibody. Only case 10 showed significant induction of apoptosis by anti-Fas antibody, which was proven by both morphologic change and DNA ladder formation.

The addition of recombinant IL-6 partially inhibited Fas-induced apoptosis in cases 10 and 13, but not in case 14 (Fig 6).

Fas-independent apoptosis of fresh samples. Cases 7, 9, 10, and 13 showed apoptosis at a rate of more than 10% during incubation with control IgM (Fig 5), which indicated that apoptosis could be induced by a Fas-independent pathway (Fas-independent apoptosis). Indeed, cases 10 and 13 showed DNA ladder formation when incubated with control IgM for 2 hours (Fig 6). As these cases tended to show high serum LDH levels (Table 1), the correlation between Fas expression and the ratio of Fas-independent apoptosis was analyzed (Fig 7). Plasma cells from Fas-positive cases showed higher Fas-independent apoptosis rates than those from Fas-negative cases, indicating higher sensitivity for apoptosis in Fas-expressing cells, although this apoptosis was not mediated by anti-Fas antibody.

DISCUSSION

AMHL is known to have a poor prognosis. Usually, the progression of AMHL is correlated with the level of serum LDH, suggesting that LDH reflects tumor mass and is derived from tumor cells. As myeloma patients usually do not have high serum LDH, AMHL must have specific mechanisms of LDH elevation. In this study, we demonstrated that tumor cells from cases with high serum LDH expressed Fas and underwent apoptosis after stimulation with anti-Fas antibody. Fas-positive plasma cells of AMHL patients may undergo apoptosis in vivo after stimulation with Fas ligand, which has been cloned recently. Fas-induced apoptosis may be found in limited cases, as induction of apoptosis by anti-Fas antibody was not significant in most cases. Although more than 90% of RPMI 8226 cells died after 24 hours of culture with anti-Fas antibody, only 13.7% of
Table 2. Profiles of Patients With Myeloma and PCL and Fas Expression on Fresh Tumor Cells

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Type</th>
<th>Stage</th>
<th>Source</th>
<th>Plasma Cells (%)</th>
<th>LDH (U/L)*</th>
<th>% Fas Expression (median fluorescence intensity)</th>
<th>VLA4 (%)</th>
<th>VLA5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fas-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>G-K</td>
<td>II A</td>
<td>BM</td>
<td>36.5</td>
<td>415</td>
<td>2.29 (2.58)</td>
<td>35.81</td>
<td>1.09</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>G-K</td>
<td>III B</td>
<td>BM</td>
<td>24.1</td>
<td>367</td>
<td>0.89 (2.48)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>BJP-K</td>
<td>III B</td>
<td>BM</td>
<td>80.4</td>
<td>437</td>
<td>5.75 (2.34)</td>
<td>46.23</td>
<td>2.99</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>A-λ</td>
<td>III A</td>
<td>PE</td>
<td>100</td>
<td>388</td>
<td>3.16 (2.65)</td>
<td>73.66</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fas-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>G-λ</td>
<td>II A</td>
<td>BM</td>
<td>47.2</td>
<td>251</td>
<td>33.00 (6.32)</td>
<td>95.56</td>
<td>20.56</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>G-K</td>
<td>I A</td>
<td>BM</td>
<td>28.0</td>
<td>398</td>
<td>34.94 (7.72)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>G-K</td>
<td>III A</td>
<td>BM</td>
<td>86.2</td>
<td>569</td>
<td>23.93 (6.48)</td>
<td>94.73</td>
<td>14.93</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>A-λ</td>
<td>II A</td>
<td>BM</td>
<td>25.1</td>
<td>154</td>
<td>32.33 (7.98)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>A-K</td>
<td>III A</td>
<td>PE</td>
<td>&gt;95</td>
<td>871</td>
<td>14.00 (5.02)</td>
<td>87.03</td>
<td>2.63</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>A-K</td>
<td>III A</td>
<td>PE</td>
<td>&gt;95</td>
<td>465</td>
<td>31.06 (6.53)</td>
<td>87.36</td>
<td>5.96</td>
</tr>
<tr>
<td>11</td>
<td>59</td>
<td>BJP-λ</td>
<td>III A</td>
<td>PE</td>
<td>&gt;95</td>
<td>397</td>
<td>48.68 (8.46)</td>
<td>99.25</td>
<td>NT</td>
</tr>
<tr>
<td>12 (PCL)</td>
<td>78</td>
<td>NS</td>
<td>III A</td>
<td>PB</td>
<td>&gt;95</td>
<td>335</td>
<td>29.26 (5.81)</td>
<td>96.26</td>
<td>9.30</td>
</tr>
<tr>
<td>13 (PCL)</td>
<td>45</td>
<td>A-λ</td>
<td>III B</td>
<td>PB</td>
<td>91.5</td>
<td>830</td>
<td>17.89 (4.76)</td>
<td>66.93</td>
<td>5.36</td>
</tr>
<tr>
<td>14 (PCL)</td>
<td>25</td>
<td>BJP-λ</td>
<td>III B</td>
<td>PB</td>
<td>90.0</td>
<td>850</td>
<td>84.53 (17.17)</td>
<td>98.29</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Fas-negative, cases with less than 10% expression of Fas; Fas-positive, cases with more than 10% expression of Fas.

Abbreviations: NS, nonsecretory; NT, not tested; G, IgG; A, IgA; BJP, Bence Jones protein.

* Normal range: 236 to 427 U/L.

Apoptosis was found after 2 hours of culture. Apoptosis must have initiated right after the addition of anti-Fas antibody and proceeded in a time-dependent manner, leading to cell death in the major cell population after 24 hours. However, our preliminary experiments showed that convincing data indicating a time-dependent increase in DNA fragmentation or morphologic change could not be obtained due to the increasing degradation of dead cells that had undergone apoptosis. This can be explained by the fact that cell death is a late event in apoptosis that is preceded by DNA fragmentation and morphologic change.

A plasma cell line, RPMI 8226, which originated from a PCL case, showed a high level of induction of apoptosis by anti-Fas antibody. As fresh cells from all PCL cases and two of five cases of myeloma showed induction of apoptosis by anti-Fas antibody, Fas expression and Fas-mediated apoptosis may be frequent in tumor cells from PCL. Although IL-6 has been reported to be a growth factor for tumor cells in PCL, apoptosis was not significantly rescued by the addition of IL-6 in case 14, and plasma cells from cases 10 and 13 showed only partial inhibition of Fas-mediated apoptosis by IL-6. IL-6-responsive tumor cells may be rescued from Fas-induced apoptosis by IL-6, although heterogeneous requirement of IL-6 by fresh myeloma cells was not examined here. Indeed, an observation that there was no inhibition of Fas-mediated apoptosis in the RPMI 8226 cell line supports the idea that IL-6 promotes cell survival in PCL cells through a different mechanism than that mediated by Fas.
Plasma cell apoptosis

Fig 5. Induction of apoptosis in fresh samples during culture with control IgM, anti-Fas antibody (CH11), or in combination with anti-Fas antibody and 1 ng/mL IL-6 for 2 hours. The ratio of apoptosis was evaluated by morphologic analysis of fresh samples stained with May-Giemsa staining as described in Materials and Methods. The ratio of cells showing typical morphologic features of apoptosis is shown.

8226 cell line, which does not respond to exogenous IL-6 (data not shown), is compatible with this hypothesis.

The detection of apoptosis was analyzed by morphologic analysis and DNA gel electrophoresis. When incubated for 24 hours with control antibody or anti-Fas antibody, the dead cell rate of fresh samples (cases 2, 7, 9, 10, and 12 through 14), which was analyzed by trypan blue dye exclusion method, ranged from 7.9% to 49.2% and 20.0% to 68.3%, respectively (data not shown). Although there was a trend toward an increasing dead cell rate with stimulation of anti-Fas antibody, confirmation of Fas-mediated apoptosis by trypan blue dye exclusion assay was not possible because of a high incidence of cell death during culture with control antibody. Therefore, 24 hours of culture may not be adequate to detect Fas-mediated apoptosis; the instability of fresh tumor cells during culture due to the dependency on several growth factors may complicate analysis of Fas-induced apoptosis. On the other hand, viable cell assays of cell lines seemed to be successful because of their stable growth in vitro.

Expression of the bcl-2 gene is reported to inhibit Fas-induced apoptosis. As found in plasma cell lines, bcl-2 expression may contribute to various sensitivity to Fas-induced apoptosis of fresh plasma cells. However, bcl-2 expression should not be the only inhibitory factor for Fas-mediated apoptosis, as KHM1B and KHM11, which were less sensitive to Fas-mediated apoptosis than RPMI 8226, did not express bcl-2. The plasma cell line KMS12PE has a
high level of expression of the cyclin gene PRAD-1 because of a translocation at chromosome 11q13,12,19 which may be responsible for the inhibition of Fas-induced apoptosis. Further analysis of this mechanism would provide useful information regarding the heterogeneous regulation of bcl-2 in controlling apoptosis of malignant plasma cells.

The plasma cell lines KMS12BM and KMS12PE were derived from the same patient’s bone marrow and pleural effusion, respectively. As KMS12BM has the phenotype of a CD20-positive plasmablast and KMS12PE has the phenotype of a CD20-negative mature plasma cell,12 Fas expression may not be induced in the plasmablast. Fas expression was not found in some cases despite a large population of tumor cells. These findings indicate that Fas gene expression is differentially regulated in each case and relatively specific for AMHL. The mechanism of heterogeneous Fas gene regulation would be very important for understanding the heterogeneity of myeloma cells.

VLA4 expression seemed to be correlated with Fas expression on fresh plasma cells. VLA4 has been reported to be useful in classifying the differentiation stages of plasma cells in combination with VLA5.20 However, VLA4 has not been reported to be differentially expressed on malignant plasma cells. If high levels of VLA4 expression are correlated with high-level Fas expression and elevated serum LDH, high VLA4 expression would be a factor of poor prognosis, although the number of studied cases is small. It is suggested that the VLA4 gene may have a similar mechanism of transcriptional regulation as the Fas gene. As VLA5 induces apoptosis in a myeloid leukemia cell line,21 VLA4 in plasma cells may also have a similar function in combination with Fas antigen. Further genetic analysis of the 5' region of Fas and the VLA4 gene would help in elucidating this point.

Interestingly, Fas-independent apoptosis was observed in Fas-positive cells from cases with high LDH. This indicates that Fas-positive cells are susceptible to apoptosis that is not mediated through Fas antigen. The Fas gene may be coactivated by unknown mechanisms, during which cells become sensitive to apoptosis. Indeed, Fas expression was slightly upregulated during apoptosis in a plasma cell line, KHM11, cultured under serum-free conditions (H.H., unpublished observation, May 1994). Fas antigen may be upregulated when cells undergo apoptosis through the Fas-independent pathway. The level of Fas-independent apoptosis was heterogeneous. This indicates that requirement of soluble factors such as IL-6 is heterogeneous in each case, although further growth analysis by addition of various cytokines was not performed. Analysis of apoptosis during culture in vitro may be a good method by which to determine the dependency of tumor cells on cytokines and/or microenvironments.

Although the mechanisms of Fas-independent apoptosis are not clear, the fresh cells obtained from cases with high LDH showed Fas expression and higher incidence of apoptosis than other cases, suggesting that plasma cells from patients with AMHL may be highly sensitive to apoptosis, thereby resulting in high levels of serum LDH. In AMHL patients, both proliferation and apoptosis may occur simulta-


Expression of Fas/Apo-1 (CD95) and apoptosis in tumor cells from patients with plasma cell disorders

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