Interleukin-6 Inhibits Fas-Induced Apoptosis and Stress-Activated Protein Kinase Activation in Multiple Myeloma Cells

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Fas belongs to the family of type-1 membrane proteins that transduce apoptotic signals. In the present studies, we characterized signaling during Fas-induced apoptosis in RPMI-8226 and IM-9 multiple myeloma (MM) derived cell lines as well as patient plasma cell leukemia cells. Treatment with anti-Fas (7C11) monoclonal antibody (MoAb) induced apoptosis, evidenced by internucleosomal DNA fragmentation and propidium iodide staining, and was associated with increased expression of c-jun early response gene. We also show that anti-Fas MoAb treatment is associated with activation of stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (MAPK); however, no detectable increase in extracellular signal-regulated kinases (ERK1 and ERK2) activity was observed. Because interleukin-6 (IL-6) is a growth factor for MM cells and inhibits apoptosis induced by dexamethasone and serum starvation, we examined whether IL-6 affects anti-Fas MoAb-induced apoptosis and activation of SAPK or p38 MAPK in MM cells. Culture of MM cells with IL-6 before treatment with anti-Fas MoAb significantly reduced both DNA fragmentation and activation of SAPK, without altering induction of p38 MAPK activity. These results therefore suggest that anti-Fas MoAb-induced apoptosis in MM cells is associated with activation of SAPK, and that IL-6 may both inhibit apoptosis and modulate SAPK activity.

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fragmentation and SAPK activity induced via Fas, without altering p38 MAPK. Our studies therefore suggest that IL-6 can modulate Fas-triggered apoptosis and SAPK activation in MM cells.

MATERIALS AND METHODS

Cell Culture

The human MM cell lines RPMI-8226 and IM9 (ATCC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO), 100 U/mL penicillin (pen), 100 mg/mL streptomycin (strep) and 2 mM L-glutamine (GIBCO, Grand Island, NY). Mononuclear cells were isolated from the peripheral blood of a patient with plasma cell leukaemia (PCL) by Ficoll-Hypaque density gradient centrifugation and incubated with HB7 (anti-CD38) monoclonal antibody (MoAb)-biotin-streptavidin and 2H4 (anti-CD45RA) MoAb-fluorescein isothiocyanate on ice. Tumor cells (96% ± 2% CD38-CD45RA-) were isolated using an Epics C Cell Sorter (Coulter Electronics, Hialeah, FL), washed, and resuspended in RPMI-1640 media containing 10% FBS, 2 mM L-glutamine, 100 U/mL Pen, and 100 U/mL Strep. Cells were treated with either medium or with 7C11 anti-Fas MoAb (1:800) for 30 minutes at 37°C before the addition of protein A-HCL Sepharose for 1 hour. Supernatants were incubated with either preimmune rabbit serum or specific antibodies for SAPK or ERK1/ERK2 (Santa Cruz Biotechnology, San Diego, CA) or p38 MAPK (provided by Dr Roger Davis, University of Massachusetts Medical School, Worcester) for 2 hours at 37°C. The reaction was incubated for 15 minutes at 30°C. Reactions were terminated by boiling in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and phosphorylated proteins analyzed by SDS-PAGE, Coomassie blue staining, and autoradiography.

Immune Complex Kinase Assays

Assays were performed as previously described.44 Lysates were precleared by incubating with 5 mg/mL rabbit-antimouse IgG for 1 hour at 4°C, followed by a 30 minutes incubation with protein A-Sepharose. Supernatants were incubated with either preimmune rabbit serum or specific antibodies for SAPK or ERK1/ERK2 (Santa Cruz Biotechnology, San Diego, CA) or p38 MAPK (provided by Dr Roger Davis, University of Massachusetts Medical School, Worcester) for 2 hours at 4°C before the addition of protein A-Sepharose for 1 hour. Immune complexes were washed three times with lysis buffer, once with kinase buffer, and resuspended in kinase buffer containing 1 μCi/mL [γ-32P]ATP (3,000 ci/mmol; New England Nuclear, Boston, MA) and GST-Jun(2-100) fusion protein for 2 hours at 4°C. GST-Jun fusion protein was prepared as previously described.43 Protein complexes were washed with lysis buffer and incubated in kinase buffer (20 mM L HEPES, pH 7.0, 10 mM MgCl2, and 10 mM L-phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 mg/mL of leupeptin and aprotinin). After incubation on ice for 30 minutes, insoluble material was removed by centrifugation at 14,000 rpm for 10 minutes at 4°C. Lysates were incubated with 5 mg immunobilized GST or GST-Jun(2-100)fusion protein for 2 hours at 4°C.

Phenotypic Analysis of MM Cells

Cells were examined for the expression of Fas Ag before and after culture for 3 to 4 hours with IL-6 (100 ng/mL) using 7C11 anti-Fas MoAb and indirect immunofluorescence and flow cytometric analysis, as in previous studies.4 Antibody-coated cells were enumerated by flow cytometric analysis using an EPICS V cell sorter (Coulter Electronics).
Anti-Fas MoAb Induces Apoptosis in MM Cells

To test for Fas-triggered apoptosis, RPMI-8226 MM cells were cultured in media alone or with anti-Fas MoAb for 3, 6, and 9 hours. DNA cleavage was analysed by agarose gel electrophoresis of the $^{32}$P-labeled genomic DNA. Low to undetectable DNA cleavage was observed in the cells cultured in media alone (Fig 1A). In contrast, exposure to anti-Fas MoAb resulted in significant cleavage of DNA into oligonucleosomal fragments of approximately 200 bp. This pattern was most prominent after 6 and 9 hour culture with anti-Fas MoAb; longer periods of exposure to anti-Fas MoAb (24 hours and 48 hours) resulted in diffuse DNA degradation. Treatment of IM-9 MM cells and PCL patient cells with anti-Fas MoAb for 6 hours and 9 hours also induced apoptosis, evidenced by DNA-ladder formation (data not shown). In contrast, incubation of RPMI-8226, IM-9, and PCL cells with an isotype matched control MoAb did not cause DNA-laddering.

Apoptosis was further analyzed by FACS analysis. Cells were treated with anti-Fas MoAb for the indicated time periods and then stained with propidium iodide; percentage of apoptotic cells was determined by flow cytometric analyses. Treatment of RPMI-8226 MM cells with anti-Fas MoAb for 48 hours induced a significant increase in percentage of apoptotic cells (73% ± 5%, three independent experiments) (Fig 1B).

Anti-Fas MoAb Induces c-jun mRNA in MM Cells

Because our previous studies showed that c-jun, an early response gene, is induced during apoptosis triggered by ara-C, we in the present study measured expression of c-jun gene during Fas-induced apoptosis in RPMI 8226 MM cells. Low to undetectable levels of c-jun transcripts were observed in cells cultured in media alone (Fig 2). In contrast, treatment of MM cells with anti-Fas MoAb induced c-jun transcript as early as 1 hour; densitometry showed that c-jun mRNA levels peaked at 6 hours (8.2 ± 0.5-fold increase) and de-
Fig 3. Anti-Fas MoAb induces activation of SAPK and p38 MAP kinase. (A) RPMI 8226 MM cells were treated with 5 μg/mL of anti-Fas MoAb for 15, 30, and 60 minutes. Cell lysates prepared from control cells (media alone) and anti-Fas MoAb-treated cells were incubated with glutathione-sepharose beads containing 5 μg of GST-Jun (2-100) for 2 hours. The resulting protein complexes were washed and then incubated in kinase buffer containing GST-Jun fusion protein and [γ-32P]ATP for 15 minutes at 30°C. The phosphorylated proteins were resolved by 10% SDS-PAGE, stained with Coomassie blue, dried, and analyzed by autoradiography. Lysates from control and anti-Fas MoAb-treated RPMI-8226 cells were also immunoprecipitated with anti-SAPK Ab (B), anti-p38 MAP kinase Ab (C), or anti-ERK-1 Ab (D). Immune complex kinase assays were performed by addition of GST-Jun (B), GST-ATF-2 (C), or MBP (D) and [γ-32P]-ATP and incubation for 15 minutes at 30°C. The phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by Coomassie blue staining and autoradiography. Anti-SAPK immunocomplexes prepared from control and anti-Fas treated IM-9 (E) and PCL (F) cells were also assayed for phosphorylation of GST-Jun.

IL-6 Inhibits Fas-Induced DNA Fragmentation in MM Cells

IL-6 triggers a twofold increase in stimulation index of RPMI-8226 MM cells. Recent studies have also shown that IL-6 prevents apoptosis in MM cells and B cell hybridomas induced by dexamethasone, factor deprivation and serum starvation11,12; therefore, we determined whether IL-6 inhibits RPMI-8226 MM cell apoptosis triggered via Fas. RPMI-8226 MM cells were cultured with IL-6 (100 ng/mL) for 3 to 4 hours before addition of anti-Fas MoAb for 6 hours; a substantial decrease in the nucleosomal DNA-laddering was evident in cells treated with IL-6 before culture with anti-Fas MoAb, compared to cells cultured in media or with anti-Fas MoAb (Fig 4C). This inhibition of apoptosis was not
Fig 4. Effect of IL-6 pretreatment on anti-Fas MoAb-induced SAPK and p38 MAPK activation, as well as DNA fragmentation, in RPMI 8226 MM cells. Cells were cultured in media alone or pretreated with IL-6 as indicated before anti-Fas MoAb treatment. Cell lysates were immunoprecipitated with anti-SAPK Ab (A) or anti-p38 MAPK Ab (B). The resulting protein complexes were washed and then incubated in kinase buffer containing GST-Jun and \( \gamma^{32}\)P]ATP for 15 minutes at 30°C. The phosphorylated proteins were resolved by 10% SDS-PAGE, stained with Coomassie blue, dried, and analyzed by autoradiography. (C) Cells were cultured in either media alone or treated with 100 ng/mL of IL-6 for 3 to 4 hours before stimulation with anti-Fas MoAb for 6 hours. Genomic DNA was isolated, end-labeled with \( \gamma^{32}\)P]ATP, and analyzed by 1.8% agarose gel electrophoresis. MW, molecular weight markers (123-bp ladders and HindIII digest of I-DNA).

**DISCUSSION**

In the present study, we characterized the signaling mechanism involved in Fas-induced apoptosis of RPMI-8226 MM cells. Anti-Fas MoAb induced DNA fragmentation, which was associated with increased c-jun transcripts, as well as increased SAPK and p38 MAPK activity. Importantly, IL-6 significantly reduced both DNA fragmentation and SAPK activity triggered via Fas. Therefore, our studies suggest that anti-Fas MoAb-induced activation of SAPK is associated with apoptosis, and that IL-6 can abrogate both SAPK activation and apoptosis.

Multiple studies have shown that triggering via Fas(APO-1) Ag induces apoptosis in various cell types. Anti-Fas Ag is a 45-kD type I membrane protein of the TNF/nerve growth factor receptor superfamily, which also includes CD40, CD30, CD27, and OX40. Fas ligand is a 40 kD type II membrane protein of the TNF family, which includes TNF-α, TNF-β, CD40 ligand, and CD30 ligand. Shima et al have previously reported that five human MM cell lines and the majority of freshly isolated patient MM cells express Fas Ag, and that anti-Fas MoAb treatment triggered loss of cell volume, membrane blebbing, fragmentation of nuclei, and condensed chromatin in some MM cell lines and MM patient samples. Hata et al have similarly found Fas Ag to be present on the majority of MM cell lines and patient cells;
anti-Fas MoAb triggered apoptosis, confirmed by morphologic analysis alone and/or DNA electrophoresis in some cases. Finally, Westendorf et al. have demonstrated apoptosis, confirmed both by DNA fragmentation and morphological changes, in some human MM cell lines and patient samples. The present studies showed anti-Fas MoAb induced fragmentation of MM cell DNA into multiples of nucleosome sized fragments, changes in chromatin structure characteristic of apoptosis or programmed cell death.

The signaling mechanism initiated by Fas is not yet delineated. Mutational studies of Fas have shown that an intracellular domain, homologous to a region of the TNF receptor, is essential for Fas-mediated cell death. However, the intracellular region of the Fas molecule has no consensus sequence for kinases or phosphatases. A possible mechanism of Fas signaling is via acid sphingomyelinase, analogous to TNF. TNF induces acid sphingomyelinase activity, resulting in increased production of ceramide which induces apoptosis. Alternatively, Fas-mediated apoptosis may involve an IL-1β converting enzyme-like protease. Recent studies which show that UV light, ionizing radiation, and in no cell lines, we showed inhibition of MM cell DNA into multiples of mRNA and BCL-x protein. In their study, very high levels cases. Finally, Westendorf et al. have demonstrated over, Schwarze and Hawley have shown that IL-6 mediated resistance to Fas-related apoptosis. Our present studies in MM cells also suggest involvement of SAPK and p38 MAPK in apoptosis. They demonstrate that anti-Fas MoAb induced both DNA fragmentation and increased c-jun mRNA levels in MM cells, as well as c-Jun phosphorylation (confirming SAPK activation) and GST-ATF2 phosphorylation (confirming p38 MAPK activation). Moreover, they further support the view that initiation and continuation of apoptosis involves activation of transcription factors, phosphorylation of c-Jun upregulates c-jun transcription through AP-1 binding, and the induction of c-jun expression observed in our study may therefore be mediated by the SAPK signaling pathway.

In previous reports, resistance of MM cells to Fas-related apoptosis has been attributed to BCL-2 expression. For example, the single MM cell line found by Hata et al to be resistant to anti-Fas MoAb treatment despite its cell surface Fas Ag expression highly expressed BCL-2. However, others have found BCL-2 expression to be similar in both Fas-sensitive and Fas-resistant MM cell lines, suggesting that alternative mechanisms may confer protection against Fas-related apoptosis. In our preliminary studies, anti-Fas MoAb treatment downregulated expression of BCL-2 in RPMI-8226 MM cells, further supporting a protective mechanism other than BCL-2. Importantly, Westendorf et al. found resistance of MM cells to anti-Fas MoAb-triggered apoptosis to be related either to the lack of cell surface Fas Ag expression; or to a soluble factor present in serum, since MM BMMC were resistant and purified MM cells were sensitive to anti-Fas MoAb treatment.

Prior studies have shown that IL-6 is one such soluble factor which can inhibit apoptosis of MM cells induced by Fas or other stimuli. Specifically, studies of MM cell lines and patient MM cells have shown that IL-6 inhibits apoptosis triggered by serum starvation or dexamethasone, but not that stimulated by doxorubicin or etoposide. Because IL-6 decreased BCL-2 expression in these MM cell lines, its anti-apoptotic effects cannot be directly related to BCL-2. Moreover, Schwarze and Hawley have shown that IL-6 mediated suppression of apoptosis in IL-6 dependent B9 cells does not involve induction of endogenous BCL-2 expression, but rather is associated with upregulation of cellular BCL-x mRNA and BCL-x protein. In their study, very high levels of BCL-2 in B9 cells, achievable only using retroviral or bovine papilloma virus-based vectors, suppressed apoptotic death induced by IL-6 deprivation. Therefore, to date, the anti-apoptotic effect of IL-6 on MM cells is not attributable to its effects on BCL-2. The importance of BCL-2 for in vivo survival of MM cells has also been questioned, since recent studies have found higher percentages of BCL-2 positive cells in patients with a long survival, compared to patients with refractory MM and short survival.

In the present study, we also show that IL-6 is one soluble factor inhibiting apoptosis of MM cells triggered by Fas. In contrast to studies by Hata et al. in which IL-6 (1 ng/mL) inhibited apoptosis in only a single MM patient sample and in no MM cell lines, we showed inhibition of MM cell apoptosis using 100 ng/mL IL-6. Inhibition of apoptosis at higher IL-6 concentrations in vitro observed in our study suggests that the increased in vivo IL-6 serum levels in patients with advanced stages of disease may not only mediate tumor cell growth, but also enhance tumor cell survival. Our finding that IL-6 pretreatment alters anti-Fas MoAb induced SAPK activity and apoptosis without altering p38 MAPK activity suggests that IL-6 may act on upstream events involved in activating SAPK, and not p38 MAPK. Previous studies in MM and other cells have shown that IL-6 can initiate growth via the Ras dependent MAPK cascade: sequential activation of Shc, Grb2, Sos, Raf, MEK, and MAPK. In addition, Billadeau et al. have also reported that expression of mutant ras in the IL-6 dependent ANBL-6 MM cell line facilitates growth in the absence of IL-6 and suppresses apoptosis. Finally, recent studies have shown that activation of ERK signaling pathways can inhibit apoptosis, and conversely, that inactivation of ERK signaling along with activation of SAPK or p38 MAPK may be critical for apoptosis. These studies, coupled with the present report, suggest that IL-6 may exert an anti-apoptotic effect in MM cells by modulating the Ras-Raf-MAPK signaling cascades. Further characterization of the mechanism whereby IL-6 inhibits apoptosis in MM cells, including abrogation of SAPK activation, may not only provide clues to basic growth control in MM, but also offer new treatment strategies.

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