Fas-Mediated Apoptosis in Ewing’s Sarcoma Cell Lines by Metalloproteinase Inhibitors

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**Background:** Fas ligand (FasL) is a transmembrane protein that induces apoptosis (programmed cell death) in susceptible cells by interacting with its receptor, Fas. Transmembrane FasL is cleaved by a metalloproteinase enzyme into a soluble form that is released into the extracellular medium. Tumors of the Ewing’s sarcoma family express functional transmembrane FasL and release soluble FasL. This cleavage is inhibited by a matrix metalloproteinase inhibitor (MMPI). We therefore hypothesized that MMPIs can lead to apoptosis of tumor cells by inducing accumulation of transmembrane FasL.

**Methods:** Ewing’s sarcoma and neuroblastoma cell lines were treated with two synthetic MMPIs (BB-3103 and A-151011) and examined for apoptosis and expression of FasL and Fas.

**Results:** Although MMPIs increase levels of FasL and Fas proteins on the surface of all tumor cells studied, they induced apoptosis in Fas-sensitive but not in Fas-resistant cell lines; the induction of apoptosis was inhibited by a Fas-neutralizing antibody. The increase in protein expression was not associated with enhanced transcription. Treatment with an MMPI sensitized the Ewing’s sarcoma cells to Fas-activating antibody and to doxorubicin-induced apoptosis.

**Conclusions:** MMPIs cause accumulation of transmembrane FasL by inhibiting its cleavage, accumulation of Fas (probably secondarily to FasL cleavage inhibition), and decreased levels of soluble FasL. These effects lead to apoptosis in Fas-sensitive cell lines. The observed cooperative action of MMPIs and doxorubicin suggests a possible role of MMPIs in combination treatments with standard apoptosis-inducing chemotherapeutic agents.

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Matrix metalloproteinases (MMPs) contribute to tumor invasion and metastasis (1), and synthetic matrix metalloproteinase inhibitors (MMPIs), such as BB-94 (batimastat) and BB-2516 (marimastat), have been suggested for clinical application to inhibit metastasis (2,3). In addition, a yet unidentified MMP-like enzyme has been implicated in the cleavage and shedding of the apoptosis (i.e., programmed cell death)-inducing Fas ligand (FasL) into the extracellular medium in a soluble form (4–8). FasL (tm-FasL) is a transmembrane protein of the tumor necrosis factor (TNF) family (9), with an amino-terminal cytoplasmic and a carboxy-terminal extracellular region (type II protein). The human soluble FasL (s-FasL) consists of the largest part of the extracellular domain of the FasL molecule. FasL induces apoptosis upon binding to Fas (also known as Apo-1 or CD95), a type I transmembrane receptor of the TNF/nerve growth factor receptor superfamily (10). Fas and FasL are involved in the regulation of the immune response and the deletion of autoreactive lymphocytes (11,12), and various carcinomas (13–15). Studies have shown that FasL is also expressed in a wide variety of tumors, including melanomas (16), astrocytomas (17), lymphomas (18–20), and various carcinomas (21–27), and helps them evade the immune system by killing Fas-expressing activated lymphocytes. The MMP-mediated cleavage of tm-FasL into s-FasL suggests that MMPs could be involved in the regulation of apoptosis.

We have recently shown that a high percentage of the Ewing’s sarcoma family tumors express Fas and biologically active tm-FasL and s-FasL. We have also shown that the shedding of s-FasL into the medium decreases substantially with MMPI treatment (28). Because co-expression of Fas and tm-FasL by Fas-sensitive tumor cells would lead to their death, we hypothesized that they may use the cleavage process to decrease their surface tm-FasL (29,30). If this hypothesis is correct, then reversing the shedding of s-FasL with an MMPI would result in accumulation of tm-FasL and cell death. We investigated this hypothesis by treating Fas-sensitive and Fas-resistant Ewing’s sarcoma family tumor/neuroblastoma cell lines with synthetic MMPIs.

**Materials and Methods**

**Cell Lines and Reagents**

The Ewing’s sarcoma family tumor cell lines SK-N-MC (American Type Culture Collection, Manassas, VA), TC-268 (31), and the two clones (CHP-100S and CHP-100L) of the CHP-100 cell line (32) that possess the characteristic for Ewing’s sarcoma family tumors, i.e., a t(11;22) (q24;q12) translocation and the resulting EWS/Fli-1 fusion gene product, as well as the neuroblastoma IMR-32 cell line (American Type Culture Collection) were used in this study. All Ewing’s sarcoma family tumor cell lines (28) and the IMR-32 neuroblastoma cell line (Poulaki V, Mitsiades N, Tsokos M; unpublished data) express FasL. Two of them (SK-N-MC and CHP-100L) are Fas sensitive, and three (CHP-100S, TC-268, and IMR-32) are Fas resistant [33; Poulaki V, Mitsiades N, Tsokos M; unpublished data]. All cells were grown in Dulbecco’s modified Eagle medium (DMEM) (BioWhittaker, Inc., Walkersville, MD) with 100 U/mL penicillin, 10 μg/mL streptomycin, and 10% fetal calf serum (FCS) (Life Technologies, Inc. [ GibCO BRL], Gaithersburg, MD), unless stated otherwise.

The MMPIs BB-3103 and A-151011 were gifts from British Biotech Pharmaceuticals Ltd. (Oxford, U.K.) and Abbott Laboratories (Abbot Park, IL), respectively. The anti-Fas antibodies CH11 and ZBA, which stimulate and inhibit Fas activity, respectively, were purchased from Panvera (Madison, WI). The isotype-matched antibody used in the ZBA experiments as a negative control was purchased from Pharmingen (San Diego, CA). Normal immunoglobulin G (IgG)-free horse serum was purchased from Life Technologies, Inc.

**Cell Survival Assay**

Cell survival was estimated colorimetrically with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) assay in all five cell lines. Cells were plated in 24-well plates and grown for 48 hours in DMEM with 10% FCS. After being washed in Hanks’ balanced salt solution, the cells were treated for 48 hours with 10, 20, or 40 μM of either MMPI in DMEM supplemented with 0.05% calf serum. Subsequently, the cells were incubated with 1 mg/mL MTT in fresh medium for 4 hours at 37 °C and
solubilized in a mixture of isopropanol and 1 N HCl (23: 2, vol/vol). Dye absorbance (A) was measured with a microplate reader at 570 nm, with the use of a reference wavelength of 630 nm. Cell death was estimated with the following formula: % specific death = [A(untreated cells) − A(treated cells)]/A(untreated cells) × 100%.

The MMPI-induced cell death over time was studied in the SK-N-MC cell line. Cells were grown in 24-well plates and treated with 10 μM BB-3103 for 12–72 hours. Cell death was estimated with the MTT assay as described above.

**Detection of FasL and Fas on the Cell Surface**

SK-N-MC and TC-268 cells that were selected for the apoptosis assays were also studied for levels of expression of Fas and FasL. The cells were cultured in 80-cm² flasks for 2 days and incubated with 20 μM BB-3103 overnight. Subsequently, the cells were biotinylated by incubation in 0.5 mg/ml sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) in phosphate-buffered saline (PBS) for 30 minutes at room temperature as previously described. The samples were cleared by microcentrifugation (14 000 rpm for 30 minutes at 4°C) and assessed for protein concentration. Biotinylated proteins, representing the cell surface proteins, were precipitated with Streptavidin–agarose (Sigma) overnight at 4°C. After being washed in PBS, the respective secondary peroxidase-labeled antibody was applied at a 1: 10 000 dilution for 1 hour at room temperature. The proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ).

**Northern Blotting for FasL and Fas**

Total RNA was denatured, subjected to electrophoresis in 1% agarose–formaldehyde gel, blotted by capillary transfer to a nylon membrane overnight, prehybridized for 4 hours at 42°C in digoxigenin–Easy Hyb (Boehringer Mannheim Biochemicals), hybridized overnight with the respective probe, and visualized with the digoxigenin luminescent detection kit (Boehringer Mannheim Biochemicals) according to the manufacturer’s instructions. Expression of β-actin was used as internal control for the quantification of RNA. For FasL detection, a digoxigenin-labeled probe, corresponding to nucleotides (nt) 668–1558 of the human FasL gene (GenBank D38122), was used. For Fas detection, a digoxigenin-labeled probe, corresponding to nt 257–1076 of the human Fas gene (GenBank M67454), was used.

**Effect of MMPIs on Fas-Induced Apoptosis**

To study the potential combined effect of MMPIs and Fas activation, we treated SK-N-MC cells with 10 or 20 μM BB-3103 or A-151011 for 18 hours in the presence or absence of a subtoxic dose (25 ng/mL) of the Fas-cross-linking antibody CH1. Cell death was evaluated with MTT as described above.

**Effect of Recombinant Soluble Fasl (rs-Fasl) on MMPI-Induced Apoptosis**

SK-N-MC cells were pretreated with various concentrations of rs-Fasl (Oncogene Research, Cambridge, MA) for 1 hour before 10 μM BB-3103 was added. Forty-eight hours later, cell death was evaluated with MTT as described above.

**Statistical Analyses**

To determine whether the combined in vitro treatments with MMPIs and CH11 or doxorubicin, as described above, had greater than additive effects, we performed a two-way analysis of variance (ANOVA), as recommended by Slinker (34). All other comparisons were carried out with the one-factor ANOVA method. All values represent the mean of the measurements obtained from quadruplicate wells. All P values reported are two-sided, and statistical significance was set at <.05.

**RESULTS**

**Effect of MMPIs on Fas-Sensitive and Fas-Resistant Tumor Cell Lines**

The MTT assay showed that two Fas-sensitive cell lines (SK-N-MC and CHP-100L) underwent cell death with BB-3103 (Fig. 1, A) or A-151011 (Fig. 1, B) at concentrations ranging from 10 μM to 40 μM. The difference between treated and untreated cells was statistically significant in all conditions (P<.001). On the contrary, no cell death was detected with this method in the Fas-resistant TC-268, CHP-100S, and IMR-32 cell lines with either MMPI (Fig. 1, A and B). The Fas-resistant SK-N-MC clone that we generated from the Fas-sensitive SK-N-MC cell line was also resistant to 10 μM (1.5% death; 95% confidence interval
Effect of a Fas-Neutralizing Antibody on MMPI-Induced Apoptosis

To further study the involvement of the Fas/FasL pathway in the MMPI-induced cell death, we treated the Fas-sensitive SK-N-MC cells with BB-3103 in the presence of 5 μg/mL of the Fas-neutralizing antibody ZB4 or an isotype-matched control antibody. ZB4 reduced the cell death that was induced by 10 μM BB-3103 from 28% (95% CI = 25.5%–30.6%) to 7.5% (95% CI = 7.3%–7.8%) (P < .001) and the cell death that was induced by 20 μM BB-3103 from 30.2% (95% CI = 29.3%–31%) to 13.3% (95% CI = 12.2%–14.5%) (P = .0076). In contrast, the isotype-matched control antibody did not inhibit the BB-3103-induced cell death (Fig. 1, D).

Apoptotic Nature of Cell Death Induced by MMPIs

(a) DNA ladder. Electrophoresis of isolated DNA from the SK-N-MC cells treated with 20 μM or 40 μM BB-3103 revealed a DNA ladder indicating apoptosis. In contrast, no DNA ladder was observed in the untreated SK-N-MC cells and in the Fas-resistant TC-268 cells after the same treatment (Fig. 1, A)

(b) DNA fragmentation ELISA. SK-N-MC cells treated with 10, 20, or 40 μM BB-3103 contained 218% (95% CI = 188%–247%; P < .001), 469% (95% CI = 415%–524%; P < .001), and 705% (95% CI = 614%–796%; P < .001) of the amount of fragmented DNA present in control cells, respectively. However, there was no increase in the amount of fragmented DNA in BB-3103-treated TC-268 cells (Fig. 1, D).

(c) TUNEL. SK-N-MC cells treated with BB-3103 or A-151011 exhibited many strongly positive nuclei (Fig. 2, D and E). Such nuclei were scarcely present in untreated SK-N-MC cells (Fig. 2, C) or in MMPI-treated TC-268 cells (Fig. 2, F–H).

(d) Electron microscopy. Apoptotic cells with nuclear chromatin and/or cytoplasmic condensation/fragmentation were numerous in the Fas-sensitive SK-N-MC cell line after treatment with BB-3103 (Fig. 2, J) or A-151011 (Fig. 2, K) but were absent in the untreated cells (Fig. 2, I). No apoptotic cells were observed in the Fas-resistant neuroblastoma cell line IMR-32 before or after treatment with either MMPI. The Fas-resistant Ewing’s sarcoma family tumor cell line TC-268 exhibited only scarce apoptotic cells after treatment with either MMPI (data not shown).

Levels of tm-FasL and Fas Protein and RNA on Cell Surface After MMPI Treatment

Overnight treatment with 20 μM BB-3103 increased the surface levels of tm-FasL in both SK-N-MC and TC-268 cells (Fig. 3, A). This finding suggests that the MMPI-induced cell death is secondary to accumulation of tm-FasL on the cell surface. In addition, we found that BB-3103 treatment (20 μM for 18 hours) increased the levels of Fas on the cell membrane of both SK-N-MC and TC-268 cells (Fig. 3, A). However, BB-3103 treatment had no effect on the levels of FasL RNA or Fas RNA (Fig. 3, B), which excluded enhanced transcription as a possible causative mechanism.

Increase in MMPI-Induced Cell Death With Time

The induction of cell death by 10 μM BB-3103 in the SK-N-MC cells increased gradually with time and peaked at around 60 hours after initiation of treatment (Fig. 3, C), supporting the importance of optimal protein accumulation for MMPI-induced cell death.

Effect of MMPIs on Apoptosis Induced by the Cytotoxic Anti-Fas Antibody CH11

The finding of increased surface Fas levels in MMPI-treated cells led us to investigate whether this increase was associated with increased sensitivity to Fas-mediated apoptosis. We found that the survival of SK-N-MC cells treated with a subtoxic dose of the Fas-crosslinking antibody CH11 (that activates Fas by inducing close proximity of multiple receptor molecules) or with 10 μM BB-3103 or A-151011 was 92.6% (95% CI = 88.2%–97%), 90.07% (95% CI = 87%–93.1%), and 80.39% (95% CI = 79.3%–81.5%), respectively. However, when CH11 and BB-3103 or A-151011 were administered simultaneously under the same conditions, they
resulted in a significantly lower survival, i.e., 35.98% (95% CI 33.9%–38.1%) and 37.2% (95% CI 34.3%–40.1%), respectively (Fig. 4, A). Similar results were obtained with the DNA fragmentation enzyme-linked immunosorbent assay (B) and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate end-labeling (TUNEL) method (C–H). With the latter method, large numbers of fluorescent apoptotic nuclei are present in the SK-N-MC cell line after treatment with BB-3103 (D, original magnification ×200) or A-151011 (E, original magnification ×240) but not in the untreated SK-N-MC cell line (C, original magnification ×250) or in the TC-268 cell line before (F, original magnification ×260) or after treatment with BB-3103 (G, original magnification ×250) or A-151011 (H, original magnification ×210). Apoptotic cells with condensed and fragmented nuclear chromatin were consistently seen by electron microscopy in the SK-N-MC cells treated with BB-3103 (J, original magnification ×2400) or A-151011 (K, original magnification ×2400) but not in the untreated cells (I, original magnification ×1530). Error bars represent 95% confidence intervals.

Effect of MMPIs on Apoptosis Induced by Doxorubicin

The survival of doxorubicin-treated cells was 68.3% (95% CI = 65.6%–71%); the survival of 10 or 20 μM BB-3103-treated cells was 93.7% (95% CI = 90.6%–96.9%) and 84.6% (95% CI: 82.3–86.9%), respectively. The survival of doxorubicin plus BB-3103-treated cells was 50.5% (95% CI = 50.2%–50.8%) and 46.3% (95% CI = 44.9%–47.8%) for 10 and 20 μM BB-3103, respectively (Fig. 4, B). A two-way ANOVA showed that BB-3103 potentiated the effect of doxorubicin in a statistically significant manner (P<.001 for both concentrations of BB-3103).

Effect of rs-FasL on MMPI-Induced Apoptosis

At high concentrations (ng/mL range), rs-FasL induced apoptosis in the SK-N-MC cell line; in contrast, at lower (subtoxic) concentrations (0.1–1 pg/mL), it protected SK-N-MC cells from death induced by BB-3103 (P<.001, Fig. 4, C). This protective effect could be due to a decrease in surface Fas through binding to rs-FasL and internalization of the complex.

DISCUSSION

The major role of MMPs in tumor biology is the breakdown of physical barriers, promoting tumor invasion and metastasis. However, recent evidence (35) suggests that MMPs may have more diverse roles, influencing tumor cell growth and possibly regulating angiogenesis. Synthetic MMPIs were designed to prevent tumor cell-induced changes in extracellular matrix and angiogenesis with the hope of achieving antitumor effects. Batimastat was found to be effective in rodent and human tumor models (36–41), and marimastat is currently in major phase III clinical trials in patients with advanced cancer (42). In this study, we show yet another anticancer property of synthetic MMPIs. Specifically, BB-3103 and A-151011 induced apoptosis in Fas-sensitive Ewing’s sarcoma family tumor cells by increasing the levels of surface Fas and FasL. Furthermore, MMPIs sensitized a Fas-sensitive tumor cell line to the chemotherapeutic agent doxorubicin, suggesting that MMPIs may have a broader therapeutic role.

Our novel finding of MMPI-induced apoptosis in tumor cells via the Fas/FasL pathway in vitro suggests that a similar mechanism may contribute to their anticancer activity in vivo and explains recent observations of apoptotic nuclei in MMPI-treated prostatic adenocarcinoma xenografts in nude mice (43). The fact that the apoptotic action of MMPIs is mediated by Fas is supported by our finding of inhibition of MMPI-induced tumor cell death by a Fas-neutralizing antibody. Fur-
Moreover, both MMPIs induced death only in tumor cell lines with a functional Fas pathway. Fas-resistant cell lines, including a Fas-resistant clone that we generated from the Fas-sensitive SK-N-MC parental cell line, were resistant to the cytotoxic action of the MMPIs. Because in a previous study (28) we had shown that MMPIs reduce the shedding of s-FasL in Ewing’s sarcoma family tumors, we hypothesized that the MMPI-induced tumor cell death in vitro was due to the accumulation of noncleaved FasL on the cell surface, as previously reported for activated T cells and FasL transfectants (5,30). In this study, we found that the MMPIs increased the surface protein levels not only of FasL but also of Fas, without affecting the messenger RNA levels. Recent work by Tanaka et al. (30) has suggested that, in some models, s-FasL may be a negative regulator of apoptosis by forming Fas–s-FasL complexes that are easily internalized and degraded, thus leading to decreased surface Fas levels. According to this concept, inhibition of FasL cleavage by MMPIs would lead to surface accumulation not only of FasL but also of Fas, as was shown in our study. In support of this hypothesis, exogenously added rs-FasL inhibited MMPI-induced cell death, possibly by decreasing surface Fas through binding and internalization.

The MMPI-induced enhancement in surface levels of Fas and FasL molecules would trigger apoptosis in a fashion similar to the previously reported autocrine suicide of activated T cells (11,12,44). In activated T cells, however, the increase in FasL is due to enhanced transcription; in contrast, in MMPI-treated Ewing’s sarcoma family tumor cells, it is due to inhibition of FasL cleavage. The need for accumulation of optimal Fas/FasL levels prior to induction of apoptosis is supported by our finding of increased apoptosis with time after MMPI treatment.

Although increased expression of Fas and FasL may induce apoptosis in Fas-sensitive Ewing’s sarcoma family tumor cells, it may not be sufficient to overcome existing inhibitory factors in Fas-resistant cells. This theory would explain why the Fas-resistant Ewing’s sarcoma family tumor cell lines did not undergo apoptosis with MMPI treatment in the present study, despite equally increased expression of Fas and FasL. In accordance with these data, previous studies have shown that overexpression of Fas renders Fas-responsive cells highly sensitive to Fas-mediated apoptosis (45), whereas it has no influence on the inherent susceptibility
of tumor cells to Fas-mediated cell death (46). The above data support the notion that the MMPIs may sensitize tumor cells to cell death induced by the anticancer drug doxorubicin and suggest that MMPs may potentially hold the promise that MMPIs may sensitize tumor cells to cell death induced by the anticancer drug doxorubicin, in particular in FasL-negative tumors, such as Ewing’s sarcoma family tumors, possibly in combination with traditional chemotherapy. Because FasL expression is restricted to a limited number of normal tissues in the human body (52–54), it is likely that the apoptosis-inducing activity of MMPIs will be accompanied by less toxicity than standard anticancer chemotherapy, which induces apoptosis in normal and malignant cells indiscriminately. Indeed, marimastat has been generally well tolerated in clinical studies, including one study in healthy volunteers (55,56).

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NOTES

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