Interleukin-12 Enhances the Sensitivity of Human Osteosarcoma Cells to 4-Hydroperoxycyclophosphamide by a Mechanism Involving the Fas/Fas-Ligand Pathway

Xiaoping Duan,1 Zhichao Zhou,1 Shu-Fang Jia,1 Michael Colvin,3 Elizabeth A. Lafleur,1 and Eugenie S. Kleinerman1,2
1Division of Pediatrics and 2Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, and 3Duke Comprehensive Cancer Center, Durham, North Carolina

ABSTRACT

Cyclophosphamide (CY) and its derivative ifosfamide are alkylating agents used to treat osteosarcoma (OS). The purpose of these studies was to determine whether alkylating agents affect the expression of Fas ligand (FasL) and whether interleukin 12 enhances the sensitivity of human OS cells to alkylating agents. 4-Hydroperoxycyclophosphamide (4-HC), the preactivated CY compound, and 4-hydroperoxydidechloroclophosphamide (4-HDC), its nonalkylating analogue, human OS LM6 cells, and a clone of cells derived by transfection with the interleukin 12 gene (LM6-#6) were used for these studies. Incubation of LM6 and LM6-#6 with 10 μM 4-HC increased the expression of FasL mRNA (2.5- and 3.0-fold, respectively). By contrast, 4-HDC, Adriamycin (ADR), cisplatin (CDP), and methotrexate (MTX) had no effect on FasL mRNA expression. Increased FasL expression after treatment with 4-HC was also demonstrated by immunohistochemistry and flow cytometry. Drug-induced FasL was functional and mediated cell death. We examined the effect of FasL up-regulation by 4-HC on LM6 and LM6-#6 cells. Flow cytometry showed that LM6-#6 cells expressed 2.2-fold more Fas than LM6 cells. Cytotoxicity of 4-HC, 4-HDC, ADR, CDP, and MTX on LM6, LM6-neo, and LM6-#6 were quantified. Colony-forming assay revealed an IC50 of 2.10 M for 4-HC in LM6-neo cells compared with 0.41 μM in LM6-#6 cells. The IC50 for 4-HDC, ADR, CDP, and MTX were not significantly different between the two cell lines. We concluded that the increased expression of Fas enhanced LM6-#6 sensitivity to 4-HC. These data indicate that Fas/FasL may be involved in the cytotoxic pathway of CY. Combining biological agents with chemotherapeutic agents that have complementary Fas/FasL pathway actions may offer new therapeutic alternatives.

INTRODUCTION

The Fas/Fas ligand (FasL) death receptor pathway has been identified as a key mediator of chemotherapy-induced apoptosis in leukemia and several solid tumors, including hepatoma (1, 2), neuroblastoma (3), metulloblastoma and glioblastoma (4), colon carcinoma (5, 6), Hodgkin’s disease, and Ewing’s sarcoma (7). Biochemical alterations in the expression of Fas and/or FasL by the tumor cells may therefore affect their sensitivity to antineoplastic agents. Indeed, several cytotoxic agents have been shown to induce the expression of FasL in certain tumors. Agents that increase Fas on the tumor cell surface may therefore enhance the sensitivity of these cells to drugs that increase FasL expression. Little is known about the effect of chemotherapy on Fas/FasL expression in osteosarcoma (OS) cells, however.

We recently demonstrated that interleukin (IL)-12, a cytokine produced by macrophages that augments the Th1 pathway, up-regulates the expression of Fas in highly metastatic human OS cells by a mechanism that is distinct from IFN-γ (8). We therefore reasoned that combining IL-12 with chemotherapeutic agents that up-regulate FasL may increase the chemosensitivity of OS cells. In this study, we demonstrate that treatment of OS cells with 4-hydroperoxycyclophosphamide (4-HC), the active metabolite of cyclophosphamide, leads to an increase in FasL mRNA and protein expression. By contrast, 4-hydroperoxydidechloroclophosphamide (4-HDC), the inactive metabolite of cyclophosphamide, Adriamycin (ADR), methotrexate (MTX), and cisplatin (CDP), all failed to induce FasL expression. Enhanced cell sensitivity was demonstrated in the presence of IL-12 and 4-HC only. These data indicate that there is a cell-specific response to various chemotherapeutic agents with respect to the up-regulation of FasL. Combining biological response modifiers targeting the Fas pathway with cytotoxic chemotherapy that targets the FasL pathway may augment the activity of certain chemotherapeutic agents against OS tumors.

MATERIALS AND METHODS

Reagents and Drugs. Eagle’s MEM, RPMI 1640, HBSS without Ca2+ or Mg2+, nonessential amino acids, sodium pyruvate, MEM vitamins, and L-glutamine were purchased from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). All reagents were free of endotoxin as determined by the Limulus amebocyte-lysate assay (sensitivity limit = 0.025 ng/ml). 4-HC and 4-HDC were synthesized as described previously (9). ADR was purchased from Adria Laboratory (Columbus, OH). MTX and CDP were purchased from Sigma Chemical Co. (St. Louis, MO).

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Requests for reprints: Eugenie S. Kleinerman, Division of Pediatrics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 87, Houston, TX 77030. Phone: (713) 792-8110; Fax: (713) 794-5042; E-mail: ekleiner@mdanderson.org.
Cell Lines. SAOS-LM6 (LM6), a human OS cell line, was developed by repetitive cycling of SAOS cells through the lungs of nude mice (10). LM6-#6 is a clone of LM6 transfected with a pcAGG plasmid containing murine IL-12 (10). LM6-neo cells are transfected with a pcAGG plasmid only. TE-85 human OS cells (11) were obtained from Dr. Reuben Lotan (University of Texas M. D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in Eagle’s MEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2× MEM vitamins, and 10% heat-inactivated (56°C for 30 min) fetal bovine serum at 56°C for cell growth and assays. Jurkat cells (the T-lymphoma cell line Jurkat-E6) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in complete medium (RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine).

Cytotoxicity Assays. Cytotoxicity was measured by the colony-forming assay (11, 12). Briefly, target cells (300 cells/well) in mid-log phase were seeded into 6-well plates (Costar Co., Cambridge, MA) and incubated at least 6 h to permit cell attachment. The cultures were exposed to medium alone and medium containing 4-HC, 4-HDC, ADR, CDP, and MTX individually for 24 h. Triplicate wells were used for each treatment group. The cultures were then washed twice with HBSS, refed with fresh medium, and incubated at 37°C in a 5% CO₂ humidified incubator for 13–14 days. At the end of this incubation period, the cultures were fixed with 10% formalin and stained with 0.04% crystal violet. Colonies, defined as focal growth of >30 cells, were counted. The survival rate was calculated by dividing the number of colonies present in drug-treated wells by the number present in the untreated control well × 100.

Cytotoxicity was also quantified by measuring [³H]thymidine incorporation as described previously (11, 12). LM-6, LM6-neo, and LM-6-#6 cells in mid-log growth phase were labeled with 5 µCi [³H]thymidine (ICN Biomedicals, Inc., Radiochemical Division, Irvine, CA) in each T-75 flask for 24 h. At end of incubation, the cells were washed twice with HBSS and then harvested by trypsinization by using 0.25% trypsin/0.02% EDTA for 1–2 min. The cells were resuspended in supplemented Eagle’s MEM. The 5 × 10⁵ cells were plated in each well of 96-well plates and treated with medium alone or medium containing 4-HC, 4-HDC, ADR, CDP, or MTX for 24 h. The cultures were then washed twice with HBSS, and viable cells were lysed with 0.1 ml of 0.1 N KOH. Radioactivity was quantified, and the percentage of cytotoxicity was calcu-

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Fig. 1 Interleukin (IL)-12 expression in LM6 and LM6-#6 cells. LM6 and LM6-#6 were fixed with acetone. IL-12 expression was determined using immunohistochemistry. LM6 cells did not express IL-12 (A), whereas LM6-#6 cells (B) showed strong expression of IL-12.

Fig. 2 Fas expression in LM6 cells (A) and LM6-#6 cells (B). Cells (5 × 10⁵) were stained with phycoerythrin (PE)-labeled anti-human Fas antibody and analyzed by flow cytometry.
lated by the following formula: percentage of cytotoxicity = \([\frac{(A-B)}{A}] \times 100\), where \(A\) is the radioactivity in cpm associated with cells treated with medium alone, and \(B\) is the radioactivity in cpm associated with cells treated with drugs individually.

**Jurkat Cell Cytotoxicity Assay.** LM6 or LM6-#6 (effector cells) were plated in T25 flasks, treated with 4HC, 4HDC, ADR, CDP, and MTX for 24 h, washed twice, and then plated in 96-well plates at 5 \(\times\) 10^3 cells/well. Jurkat cells (target cells) were labeled with \(^{51}\)Cr sodium chromate (100 \(\mu\)Ci/1 \(\times\) 10^6 cells) for 1–2 h at 37°C in RPMI 1640. After they were washed, the \(^{51}\)Cr-labeled Jurkat cells were cocultured with the effector cells at E:T ratios of 4:1 for 20 h at 37°C in a 5% CO_2 incubator. The supernatants were harvested, and \(^{51}\)Cr-release quantified in a gamma counter. Spontaneous release was determined from wells containing medium alone. Maximum release was determined from wells to which 1% NP40 (Sigma Chemical Co.) was added. All cultures were set up in triplicate. Lysis (percentage) was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) \(\times\) 100.

**Reverse Transcriptase-PCR.** Total RNA was isolated from the LM-6 and LM6-#6 cells by using TRIzol Reagent (Invitrogen, Carlsbad, CA). FasL expression was detected by reverse transcriptase-PCR as described previously (13, 14). One \(\mu\)g of total RNA was reverse transcribed in 20 \(\mu\)l of reverse transcriptase solution (Invitrogen). PCR amplification was carried out with 5 \(\mu\)l of the reverse transcription reaction mixture as a template. The primers for FasL for the first-step amplification PCR were as follows: FasL sense, 5'-CGAGCGAGGTTCAATTTGCTAT-3'; and FasL antisense, 5'-GACAGCAGGAAAGGGTCTGACTGC-3'. For the second, nested round PCR were

**Fig. 3** Fas ligand (FasL) expression in LM6 cells (A) and LM6-#6 cells (B) after treatment with various chemotherapeutic agents. Total RNA was isolated from 10^6 LM6 and LM6-#6 cells after treatment with various chemotherapeutic agents for 24 h. Reverse transcriptase-PCR was performed with primers specific for the human FasL receptor. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control for loading.

**Fig. 4** 4-Hydroperoxycyclophosphamide (4-HC) increases the cellular expression of Fas ligand (FasL). LM6 cells (5 \(\times\) 10^6; A) and LM6-#6 (5 \(\times\) 10^6; B) were treated with medium or 4-HC for 24 h and then stained with biotinylated antibody against human FasL, treated with streptavidin-phycoerythrin (PE), and analyzed by flow cytometry.
IL-12 Enhances OS Cells to Cytoxan via Fas/FasL Pathway

LM6-#6 cells were incubated together with 51 Cr-labeled Jurkat cells were harvested and cytotoxicity quantified. FasL sense, 5'-GGTTCTGGTTGCCTTGGTAGGATTG-3'; FasL antisense, 5'-AGCCGAAAAACGTCTGAGATTC-3'. In both amplification procedures, 35 cycles of denaturation for 30 s at 95°C, annealing for 60 s at 45°C, and extension for 2 min at 72°C were used. Samples were analyzed on a 1% agarose-Tris gel stained with ethidium bromide.

Fluorescence-Activated Cell Sorter Staining and Flow Cytometry. Indirect staining and flow cytometric analyses were carried out as described previously (8). For staining of Fas, cells were suspended in fluorescence-activated cell sorting buffer (PBS containing 2% fetal bovine serum and 0.1% sodium azide) and incubated for 30 min at 4°C with phosphotidylethanolamine-labeled anti-human Fas antibody (clone DX2; BD PharMingen, San Diego, CA). Samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA). For detection of intracellular FasL, cells were permeabilized with saponin, detached with 5 mM EDTA, washed, and then resuspended in fluorescence-activated cell sorting buffer and incubated with FasL antibody or mouse IgG1 as an isotype control for 30 min at 4°C. Cells were washed, incubated with phycoerythrin-labeled antimouse IgG for 30 min at 4°C, washed twice, and analyzed by flow cytometry.

Immunohistochemical Detection of FasL and IL-12. The LM6 cells treated by 4-HC, 4-HDC, or medium alone were fixed on slides using acetone buffer. The sections were stained using immunohistochemistry methods as described previously (10, 15) with mouse antihuman Fas-L antibody (dilution, 1:200; BD PharMingen) or rat antimonos IL-12 antibody (dilution, 1:100; Biosource International, Inc., Camarillo, CA). Secondary antibodies used were goat antimouse IgG antibody (1:200) or goat antirat IgG antibody (1:200). After three rinses with PBS, the sections were incubated with 3,3'-diaminobenzidine substrate for 5–10 min. The sections were rinsed with distilled water and counterstained with Gill’s hematoxylin.

Statistical Analysis. Data were analyzed by the Student’s t test, and values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of IL-12 on Fas Expression. We have previously demonstrated that IL-12 up-regulates Fas expression on OS cells (8). The presence of IL-12 in LM6-#6 cells (a clone of LM6 cells transfected with the IL-12 gene) was confirmed using immunohistochemistry. As shown in Fig. 1, immunoreactivity for IL-12 was seen only in LM6-#6 cells. Cell surface Fas expression on LM6 and LM6-#6 cells was compared by fluorescence-activated cell sorting analysis. As shown in Fig. 2, 80.5% of the LM6-#6 cells were Fas positive compared with 36.2% of the LM6 cells. These data confirm our previous findings that IL-12 up-regulates Fas expression in OS cells (8) and that IL-12 transfection can lead to a permanent increase in cell-surface Fas.

Effect of Chemotherapeutic Agents on FasL Expression. We next determined the effect of various chemotherapeutic agents on FasL expression in both LM6 and LM6-#6 cells. We used 4-HC and 4-HDC as the active and inactive metabolites of cyclophosphamide. FasL expression was measured by reverse transcriptase-PCR after incubation of cells with 4-HC, 4-HDC, ADR, CDP, or MTX. Incubation of LM6 and LM6-#6 cells with 4-HC, 4-HDC, ADR, CDP, or MTX increased FasL expression by 2.5- and 3-fold, respectively (Fig. 3). This increase in FasL expression was dose dependent as incubation with 2 μM 4-HC induced a 1.7- and 1.9-fold increase in LM6 and LM6-#6, respectively. By contrast, 4-HDC, ADR, CDP, and MTX had no significant effect on FasL mRNA in either cell line (Fig. 3). To detect intracellular FasL protein expression, cells were permeabilized with saponin and then flow cytometry was performed. In LM6 and LM6-#6 cells, incubated with 4-HC FasL expression increased from 44.6 to 73.4% and from 41.7 to 67.0%, respectively (Fig. 4). Additionally, LM6 and LM6-#6 cells treated with 4-HC showed more intense immunoreactivity than the cells untreated and treated with 4-HDC (data not shown). Taken together, these data indicate that both the RNA and protein expression of FasL were selectively induced after cellular exposure to the active metabolite of cyclophosphamide.

FasL Induced by 4-HC Is Functionally Active. We next investigated whether the FasL induced after 4-HC treatment was functional and able to mediate cell death in FasL-sensitive cells. We used Fas-positive Jurkat cells as the target cells.
cells for this purpose as described previously (16). As shown in Fig. 5, 51Cr-labeled Jurkat cells showed an increased sensitivity to both LM6 or LM6-#6 cells that had been incubated with 4-HC. ADR, CDP, MTX, and 4-HDC treatment of LM6 and LM6-#6 had little or no effect on Jurkat cell killing.

**Effect of IL-12 on LM6 Sensitivity to Chemotherapeutic Agents.** We have demonstrated that IL-12 up-regulated Fas expression and that LM6-#6 had increased cell surface Fas compared with the parental LM6 cells (Figs. 1 and 2). On the basis of our results that 4-HC up-regulated the expression of FasL, we hypothesized that 4-HC would induce greater cytotoxicity in the LM6-#6 cells owning to the increased expression of Fas. Cytotoxicity was quantified by both the colony-forming assay and the [3H]thymidine assay. As shown in Figs. 6 and Fig. 7 and Table 1, LM6-#6 cells were more sensitive to 4-HC than LM6 and LM6-neo cells by both assays. The IC_{50} of 4-HC for LM6-#6 was 0.41 versus 2.10 μM for LM6-neo cells by colony-forming assay (P < 0.01). By contrast, the sensitivity of LM6-#6 and LM6-neo cells to 4-HDC, ADR, CDP, and MTX was not significantly different.

IL-12 also increased the expression of Fas on TE-85 human OS cells (data not shown). As seen with the LM6 cells, combining 4HC + IL-12 induced greater cytotoxicity in TE-85 cells than 4-HC alone. Incubating TE-85 cells with 10 μM 4-HC for 24 h induced 50% cytotoxicity compared with 85% cytotoxicity induced by 4HC + 2 ng/ml recombinant murine IL-12. TE-85 cells were not sensitive to IL-12 alone or 4-HDC with or without IL-12 (data not shown).

**DISCUSSION**

The present study demonstrated that biological agents such as IL-12 may, in addition to stimulating immune responses, also alter the biology of certain tumor cells, thus enhancing their sensitivity to chemotherapy. IL-12 is a known Th1 cytokine that augments natural killer cell activity, facilitates the induction of cytotoxic T cells, and stimulates the production of IFN-γ (17–20). These activities, plus its ability to inhibit angiogenesis (21), have been implicated in IL-12’s antitumor activity.

We recently demonstrated that IL-12 up-regulated cell surface Fas expression in OS and breast cancer cells by a mechanism that is independent of IFN-γ (8). This finding suggested that this particular activity of IL-12 could be exploited to enhance tumor cell sensitivity to chemotherapy. Numerous chemotherapy agents have been shown to mediate cell death via a mechanism involving the Fas/FasL pathway (22–27). Up-regulation of FasL has been shown to be a key element in the ability of many chemotherapeutic agents and γ-irradiation to induce apoptosis (24, 25, 27). Induction of FasL mediates apoptosis via an autocrine paracrine loop by cross-linking cell surface Fas.
Therefore, cells with higher Fas expression would be expected to be more sensitive to agents that up-regulate FasL. Similarly, combining biological agents that up-regulate cell-surface Fas with chemotherapeutic agents that up-regulate FasL should result in more efficient cell kill, thus augmenting drug-induced cytotoxicity. Conversely, down-regulation of Fas may confer tumor cell resistance to the cytotoxic agent.

In our study, the active metabolite of cyclophosphamide, 4-HC, induced the expression and protein production of FasL (Figs. 3 and 4). FasL induced after drug treatment was functional and capable of inducing cell death (Fig. 5). When cell surface Fas was increased by IL-12, these cells became even more sensitive to 4-HC (Figs. 6 and 7, Table 1). This up-regulation of FasL expression was unique to 4-HC because treatment of cells with ADR, CDP, MTX, or the inactive cyclophosphamide metabolite 4-HDC resulted in no change in FasL and no alteration in cell sensitivity in the presence of IL-12.

Fas/FasL interactions have been identified as a key pathway for mediating chemotherapy-induced apoptosis (22–27). Our findings together with other previously published studies have many implications for the drug sensitivity and resistance of different types of tumor cells. Firstly, combining immunotherapeutic agents that up-regulate Fas expression with chemotherapeutic agents that augment FasL may be an effective therapeutic approach. Before undertaking this approach, it is essential to recognize that not all tumors respond similarly to specific chemotherapy agents with regard to the induction of FasL. For

<table>
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<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>LM6</th>
<th>LM6-#6</th>
<th>LM6-neo</th>
<th>P</th>
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<tr>
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<td>0.41 μM</td>
<td>2.10 μM</td>
<td>P &lt; 0.01</td>
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<tr>
<td>4-Hydroperoxydidechloroclophosphamide (4-HDC)</td>
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<td>5.16 μM</td>
<td>6.11 μM</td>
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<tr>
<td>Methotrexate (MTX)</td>
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<td>71.2 μM</td>
<td>78.6 μM</td>
<td>P &gt; 0.5</td>
</tr>
<tr>
<td>Cisplatin (CDP)</td>
<td>0.15 μM</td>
<td>0.10 μM</td>
<td>0.12 μM</td>
<td>P &gt; 0.1</td>
</tr>
<tr>
<td>Adriamycin (ADR)</td>
<td>11.90 μM</td>
<td>12.1 μM</td>
<td>10.67 μM</td>
<td>P &gt; 0.1</td>
</tr>
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Table 1 Cytotoxic activity of 4-hydroperoxycyclophosphamide (4-HC).
example, ADR, MTX, and CDP have all been shown to up-regulate FasL on leukemia (28), neuroblastoma (3, 23), hepatoma (1), colon carcinoma, small cell lung cancer, Ewing’s sarcoma, and brain tumor cells (24, 26) but not breast cancer or renal carcinoma cells (24). We were also unable to demonstrate changes in FasL expression in our OS cells after treatment with ADR, MTX, or CDP. Thus, induction of FasL may be tumor cell specific and cautions that the universal use of combination therapy aimed at the Fas/FasL pathway may not be appropriate or effective against all solid tumors.

In summary, we have demonstrated that IL-12 up-regulates Fas expression on OS cells and that when it is combined with a chemotherapeutic agent that up-regulates FasL, cell sensitivity is increased. Thus, the Fas/FasL pathway may play a role in determining sensitivity or resistance of OS cells to chemotherapy, which in turn may have implications for developing novel biochemotherapy strategies for the management of this disease.

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