Interleukin-12 Up-Regulates Fas Expression in Human Osteosarcoma and Ewing’s Sarcoma Cells by Enhancing Its Promoter Activity

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Abstract
Interleukin-12 (IL-12) has shown significant antitumor activity in several preclinical animal tumor models. Our previous studies showed that IL-12 inhibited tumor growth in human osteosarcoma and Ewing’s sarcoma animal model. Decreased Fas expression in osteosarcoma increased the lung metastatic potential. In this study, we further examined the mechanism of IL-12 antitumor activity and showed that IL-12 significantly increased Fas expression in both human osteosarcoma cells LM7 and Ewing’s sarcoma cells TC71. Up-regulation of Fas expression increased their sensitivity to Fas-induced cell apoptosis. Constructs of the Fas promoter linked to a luciferase reporter gene were used to determine the promoter activity. IL-12 increased Fas promoter activity 4.2- and 4.9-fold in TC71 and LM7 cells, respectively. Time course studies have shown that recombinant IL-12 stimulated Fas promoter activity at 2 hours, reached the peak level at 4 hours, and then declined at 24 hours. To investigate whether IL-12 specifically enhanced Fas promoter activity, we determined whether another gene (E1A) was able to stimulate Fas promoter activity. We also evaluated effect of IL-12 on the topoisomerase IIα promoter. The results indicated that E1A but not IL-12 stimulated topoisomerase IIα promoter activity. E1A failed to increase Fas promoter activity. We also found that c-fos element at position −295 to −286 in Fas promoter was essential for IL-12-induced activation, and nuclear factor-κB transcription factor was activated after IL-12 treatment in TC71 cells. These results indicate that IL-12 up-regulates Fas expression in human osteosarcoma and Ewing’s sarcoma by enhancing Fas promoter activity.

Understanding this mechanism may lead to new therapeutic approaches for the treatment of sarcoma involving the use of IL-12.

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Introduction
Osteosarcoma and Ewing’s sarcoma are the most common primary bone tumor in children and young adults. Most patients have lung metastasis at diagnosis. The survival rate has not significantly improved in past 15 years despite of the use intensive chemotherapy in conjunction with surgery (1). Treatment of lung metastases provides the greatest challenge. Therefore, identifying new therapeutic target is a high priority. Loss of Fas expression has been implicated in tumor progression of several cancers (2, 3). We have shown previously that the levels of Fas expression inversely correlated with the lung metastasis potential of human osteosarcoma cells (4). Cells with high Fas expression did not induce lung metastases following intravenous injection, whereas those with low Fas expression formed multiple metastatic lesions in lung. Therefore, the corruption of the Fas/Fas ligand (FasL) pathway may allow tumor cells to evade host defense mechanisms in the lung, decrease tumor cell elimination, and increase their metastatic potential. Therefore, agents that enhance Fas expression may be useful for treatment of sarcoma lung metastasis.

One potential modulator of Fas expression is the cytokine interleukin-12 (IL-12), a key component in numerous immune functions, including the stimulation of T and natural killer cells. IL-12 regulates cell adhesion molecules and promotes the production of IFN-γ by T and natural killer cells (5, 6). IL-12 is a heterodimer composed of both p35 and p40 subunits. The smaller subunit is required for signaling through the IL-12 receptor, whereas the larger subunit facilitates protein binding (7). The demonstration of significant antitumor activity in several preclinical animal models has piqued interest in the therapeutic use of IL-12 (8-10).

We have shown previously that IL-12 inhibited the metastatic potential of osteosarcoma cells and decreased the tumor size of the lung metastases in nude mouse model (11). Transfection of IL-12 into highly metastatic osteosarcoma cells with low Fas expression augmented cell surface Fas expression to the levels that were similar to those in the low metastatic cells. The up-regulation of Fas by IL-12 was also shown in MDA-231 breast cancer cells, indicating that up-regulation of Fas by IL-12 was not tumor specific. Cell surface Fas expression induced by IL-12 was functional and able to mediate cell death (12).
The mechanism for this up-regulation of Fas by IL-12 is not well understood. In the present studies, using constructs of the Fas promoter linked to the firefly luciferase reporter gene, we found that IL-12 increased the activity of the Fas promoter. This increase in Fas promoter activity could not be duplicated by constructs consisting of genes for E1A and β-galactosidase (β-gal). The studies using the Fas promoter mutant vector showed that the β-Sp1 element was required for IL-12-induced activation of Fas promoter. Nuclear factor-κB (NF-κB) transcription factor was also activated after IL-12 treatment in TC71 cells. These data indicate that the ability of IL-12 to increase the cell surface Fas expression is specifically mediated by the activation of Fas promoter.

Results

IL-12 Increased Fas Expression in TC71 Ewing’s Sarcoma Cells and LM7 Osteosarcoma Cells

Northern blot analysis was shown in Fig. 1. Transfection of IL-12 gene into TC71 cells using Ad-murine IL-2 (mIL-12) effectively increased IL-12 RNA expression (both p40 and p35 subunits) and consequently enhanced Fas expression compared with TC71 control cells. In contrast, Ad-β-gal had no effect on IL-12 or Fas RNA expression in these cells. These results suggest that the up-regulation of Fas transcription was related to IL-12 expression in TC71 cells. These data were further confirmed by the reverse transcription-PCR analysis shown in Fig. 2A. Fas expression was increased 3.1-fold in LM7 osteosarcoma cells and 3.4-fold in TC71 Ewing’s sarcoma cells following transfection with Ad-mIL-12 compared with untreated control cells. By contrast, Ad-β-gal did not significantly alter Fas expression in these cells.

To detect Fas expression on the protein level, flow cytometry analysis was done. The population of Fas-positive cells was increased from 5% in untreated control cells to 40% in Ad-mIL-12-treated cells. Ad-β-gal did not significantly change Fas expression (Fig. 2B). Taken together, these data indicated that IL-12 stimulated Fas expression at both RNA and protein levels in these cell lines.

IL-12 Stimulated Fas Promoter Activation in Ewing’s Sarcoma Cells and Osteosarcoma Cells

To explore the mechanism by which IL-12 up-regulates Fas expression, we determined the effect of IL-12 on the Fas promoter linked to a luciferase reporter gene. As shown in Fig. 3A, treatment of TC71 cells with Ad-mIL-12 increased Fas promoter activity 4.2-fold when compared with control cells. However, Ad-β-gal treatment did not significantly change Fas promoter activity, suggesting that the adenoviral vector itself was not responsible for the promoter stimulation. This effect was not specific for TC71 cells. Similar results were obtained with LM7 osteosarcoma cells. Fas promoter activity was increased 4.9-fold following transfection of the IL-12 gene into LM7 cells (Fig. 3B) but not altered following treatment with Ad-β-gal. These data indicate that IL-12 up-regulates Fas expression by activating the Fas promoter.

Time Course of IL-12-Induced Activation of Fas Promoter

Recombinant IL-12 protein was used to stimulate TC71 or LM7 cells and study the time course of its activity. Fas promoter activity was enhanced 3-fold at 2 hours after treatment.
in TC71 cells and reached the peak level at 4 hours followed by a gradual decline and returned to the baseline level at 24 hours. A similar time course was observed in LM7 cells (Fig. 4).

Effect of IL-12 on Fas Promoter Activity Is Specific

To determine whether IL-12 effect on the Fas promoter is specific, we investigated if other genes (E1A and IFN-α) were able to stimulate Fas promoter activity. E1A is a tumor suppressor gene that is unrelated to IL-12 and its pathways. Previous studies have shown that E1A specifically enhanced topoisomerase IIα promoter activity (13). The data in Fig. 5 indicate that transfection of the E1A gene into LM7 cells using Ad-E1A enhanced topoisomerase IIα promoter activity but did not affect Fas promoter activity. By contrast, Ad-mIL-12 enhanced Fas promoter activity but did not change topoisomerase IIα promoter activity. Treatment of LM7 cells with recombinant human IFN-γ also failed to alter Fas promoter activity (Fig. 5A). These data suggested that the effect of IL-12 on the Fas promoter activity was specific.

FIGURE 3. IL-12 enhanced Fas promoter activity in TC71 and LM7 cells. TC71 cells (A) or LM7 cells (B) were transfected with 1 μg FPR1-Luc vector together with 1 μg Renilla luciferase vector (pRL-TK) using FuGene 6 transfection reagent. Six hours later, the cells were treated with Ad-mIL-12, Ad-B-gal, or PBS as control for 48 hours. Cells were harvested and lysed. Luciferase activity was quantified with a luminometer using Dual-Luciferase Reporter Assay System. The relative luciferase activity for each sample was adjusted with an internal control. Each measurement was done in triplicate. Columns, mean of three different experiments; bars, SD.

NF-κB Transcription Factor Is Activated after IL-12 Treatment in TC71 Cells

To determine the effects of IL-12 on NF-κB and SP1 transcription factor activity, the specific ELISA-based transcription factor activation assay (TransAM assay, Active Motif, Carlsbad, CA) was done. As shown in Fig. 7, NF-κB transcription factor activity increased 2-fold after IL-12 treatment in TC71 cells compared with the untreated control cells (Fig. 7A). These results indicate that the NF-κB transcription factor was activated following IL-12 treatment.

xB-Sp1 Element Is Required for IL-12-Induced Activation of Fas Promoter

We next investigated what specific element in Fas promoter is essential for its activation following the IL-12 treatment. xB-Sp1 motif (-295 to -286) in the Fas promoter is an important enhancer element that is the binding region for NF-κB or SP1 transcription factors. We transfected the Fas promoter truncated mutant vector Δ5-M5.7-Luc, which has a mutation of Fas promoter at the xB-Sp1 element (GGG→CCC at -295 to -293 region), into TC71 cells. The corresponding wild-type vector Δ5-Luc plasmid was used for comparison (14). The luciferase activity was measured and shown in Fig. 6. Fas promoter activity was significantly increased following the treatment with Ad-mIL-12 in Δ5-Luc truncated wild-type transfected cells. By contrast, treatment with Ad-mIL-12 did not enhance the Fas promoter activity in the mutant Δ5-M5.7-Luc-transfected cells. These results indicate that the xB-Sp1 element in this region is essential for the activation of the Fas promoter. These findings further support the previous studies in Jurkat T cells that indicate the activation of the Fas promoter required xB-Sp1 element (14).
evidence that in lymphocytes. An increasing number of studies provide the Fas expression in osteosarcoma cells may be different from that also suggest that the pathway by which production by SAOS-2, LM6, and LM7 cells (12). These results shown previously that pathway was specific to regulation was specific to pathways that differ than those seen in immune cells (15). Additionally, we have shown previously that IL-12 up-regulated Fas expression in MDA-231 breast cancer cells (12).

The previous studies have shown that activation-dependent transcriptional regulation of the human Fas promoter requires NF-κB p50-p65 recruitment. The κB-Sp1 element at the −295- to −286-bp region of the Fas promoter is essential for its activation in T cells (14). We used a truncated mutant Fas promoter vector to show that IL-12 was unable to stimulate the Fas promoter activity in tumor cells when this region was mutated. These data indicate that this κB-Sp1 motif is important for IL-12-induced activation of the Fas promoter. We further showed that NF-κB transcription factor was activated following IL-12 treatment. These data suggest that binding of NF-κB to the κB-Sp1 element during IL-12-driven Fas promoter activation is critical. It was shown that SP1 was bound to the κB-Sp1 element during normal basal transcription; however, NF-κB occupied the κB-Sp1 site during activation-driven Fas promoter induction. Binding of SP1 versus NF-κB to the κB-Sp1 element site is mutually exclusive (14). Our present studies are consistent with these observations and show that NF-κB but not SP1 is activated following IL-12-stimulated cells. Because we have shown previously the importance of the Fas/FasL pathway in the growth and development of osteosarcoma lung metastases, the identification of the essential role of the κB-Sp1 element on activation of the Fas promoter may lead to the new therapeutic approaches for osteosarcoma lung metastases.

IL-12 has shown significant antitumor activity in several preclinical models (8, 16). Despite initial enthusiasm for IL-12 as a potential antitumor compound, some severe toxic effects from systemic IL-12 therapy were reported in early clinical trials and have limited its subsequent use. Up-regulation of Fas on normal cells as well as tumor cells may contribute to these toxic complications. Understanding the diverse actions of IL-12 in normal and malignant cells may aid in strategies to use this cytokine more effectively while avoiding the systemic toxicity. Here, one additional activity of IL-12 on tumor cells has been illustrated (i.e., its ability to up-regulate Fas expression via a direct effect on promoter activity).

We have shown previously a critical role for Fas in the metastatic potential of osteosarcoma cells. Fas expression was shown to inversely correlate to metastatic potential in a nude mouse model and manipulation with the Fas expression may change metastatic activity of tumor cells (4, 17). The up-regulation of Fas expression on tumor cells may be one of the mechanisms involved in the elimination of the tumor cells via Fas/FasL-mediated apoptosis. Agents, such as IL-12, which increase Fas expression on tumor cells, may therefore help eliminate lung tumor metastases and promote tumor regression. Treatment with IL-12 reduced the number of metastatic lung nodules in nude mice injected with LM7 osteosarcoma cells (10, 11). This report shows that the antitumor activity of
IL-12 against LM7 lung metastases may mediate to activate the Fas promoter, resulting in increased Fas expression on cell surface, which in turn increases the susceptibility of cells to constitutive FasL in the lung.

Indeed, we have shown that the up-regulation of Fas expression in osteosarcoma cells via transfection with Fas plasmid inhibited their metastatic potential (17). Immunohistochemical analysis of Fas expression in patient samples indicated that Fas levels were negligible in lung metastases of osteosarcoma. Because Fas triggers cell death on binding to FasL, and the lung is one of the few organs that constitutively express FasL (18), the Fas-mediated pathway may contribute to the metastatic potential of osteosarcoma and its response to treatment. We have shown that treatment of mice bearing established osteosarcoma pulmonary metastases with IL-12 gene therapy resulted in the induction of Fas expression in treated metastases tumors and significantly inhibited the metastases growth in lungs. These results together with our previous findings show that IL-12 antitumor activity against lung metastases is mediated by activation of the Fas promotor followed by the up-regulation of the Fas receptor expression on tumor cell surface, which in turn increases cell susceptibility to constitutively expressed FasL in lungs.

Materials and Methods

Cell Line

LM7 is a human osteosarcoma cell line with high potential in lung metastases as described previously (19). TC71 is human Ewing’s sarcoma cell line. The LM7 and TC71 cells were cultured in Eagle’s modified essential medium with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1× nonessential amino acid, and 2× MEM vitamin solution (Life Technologies, Inc., Grand Island, NY). This cell line was screened with a Mycoplasma Plus PCR Primer Set (Stratagene, La Jolla, CA) and found to be free of Mycoplasma.

Recombinant Adenoviral Vectors

An adenoviral vector (Ad-mIL-12) carrying the cDNA of both p35 and p40 subunits of mIL-12 were constructed and purified as described previously (8, 20). The control adenovirus, Ad-β-gal, is an adenovirus type 5 that lacks E1A, E1B, and E3 but contains β-gal. Ad-E1A is an adenovirus type 5–based vector that contains E1A but lacks E1B and E3. All these recombinant replication-deficient adenoviral vectors were propagated in human embryonic kidney 293 cells. The viruses were purified twice by cesium chloride gradient ultracentrifugation and then dialyzed and titrated using standard methods. Cells in logarithmic growth phase were infected with adenovirus at 10 plaque-forming units per cell for 48 hours and then treated as indicated for the various experiments.

Figure 6. The nB-Sp1 element is required for IL-12-induced Fas promoter activity. Left, Δ5-Luc is a truncated (−460 to −19 bp) vector containing an essential sequence for activation of Fas promoter. Δ5-M5.7-Luc is a mutant truncated Fas promoter vector, which has GGG→CCC mutation at nB-Sp1 element (at −295 to −293 region). Right, TC71 cells were transfected with a mutant Δ5-M5.7-Luc vector or its corresponding wild-type Fas promoter vector Δ5-Luc with a Renilla luciferase vector pRL-TK. Six hours later, the cells were treated with or without Ad-mIL-12. Cell were harvested and lysed in 48 hours. Luciferase activity was measured for each sample. Columns, mean of three different experiments; bars, SD.

Figure 7. IL-12 stimulated NF-κBp65 but not SP1 transcription factor activation. NF-κBp65 (A) or SP1 (B) activity was determined by ELISA-based TransAM transcription factor activation assay kit as described in Materials and Methods. TC71 cells were treated with PBS (as control), Ad-mIL-12, or Ad-β-gal. Nuclear proteins were extracted from cells and nuclear extract (20 μg) was used in each assay. Columns, mean of three different experiments; bars, SD.
We elected to use mIL-12 gene in our previous in vivo therapy studies in nude mice (11, 16), because human cells indeed respond to mIL-12 (21). For consistency, we continued these in vitro mechanistic studies with the mIL-12. The virus titers were determined by plaque assay. Recombinant mIL-12 protein was obtained from BD Biosciences (Palo Alto, CA).

**Northern Blot Analysis**

Cells (5 \( \times 10^6 \)) were treated with Ad-mIL12 or Ad-\( \beta \)-gal or PBS (as control) at 10 plaque-forming units per cell for 48 hours. Total RNA was extracted from cells using Trizol reagent (Life Technologies, Grand Island, NY). RNA (20 \( \mu \)g) was electrophoresed on 1% formaldehyde/agarose gel and transferred to a Hybond-N\(^+\) membrane. Fas, IL-12-p40, IL-12-p35, and glyceraldehyde-3-phosphate dehydrogenase probes were labeled using the rediprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Flow Cytometry**

Cells (2 \( \times 10^6 \)) were plated in six-well plates overnight. Cells were collected and incubated with phycoerythrin-conjugated mouse anti-human Fas antibody (clone DX2, BD Biosciences) or isotype-matched phycoerythrin-conjugated mouse anti-human IgG1 antibody as control (Sigma Chemical Co., St. Louis, MO). Samples were analyzed by a FACScan (Becton Dickinson, Mountain View, CA).

**Reverse Transcription-PCR**

TC71 or LM7 cells were treated with Ad-mIL12 or Ad-\( \beta \)-gal or PBS as control for 48 hours. Total RNA was extracted from the different cells. The cDNA was synthesized using a specific truncated (containing the sequences from 690 to 19) Fas promoter vector Δ5-Luc, and its corresponding mutant (at −295 to −293 region, GGG→CCC) Fas promoter vector Δ5-M5.7-Luc were constructed as described previously (14) and kindly provided by Dr. Laurie B. Owen-Schaub (University of California-Riverside). TC71 or LM7 cells were plated in six-well plates at 2 \( \times 10^5 \) per well. After 24 hours, fresh medium was replaced. Cells were transfected with 1 \( \mu \)g of the FPR1-Luc vector or the other proper vectors with 1 \( \mu \)g Renilla luciferase vector pRL-TK by using FuGENE 6 transfection reagents (Roche Diagnostics Corp., Indianapolis, IN). Six hours later, the cells were treated with Ad-mIL12, Ad-E1A, or Ad-\( \beta \)-gal. Cells were harvested, lysed in 1× Passive Lysis Buffer (Promega), and gently shaken on a platform shaker for 15 minutes at room temperature. The cell lysates (20 \( \mu \)L) were then transferred into microcentrifuge tubes. Luciferase activity was quantified with a luminometer in a Dual-Luciferase Reporter Assay System. The relative luciferase activity for each sample was adjusted with an internal control for Renilla luciferase activity. Each measurement was done in triplicate. The mean and SD for each sample were calculated from three different experiments.

**Transcription Factor Activation Assay**

TC71 cells were treated with Ad-mIL12, Ad-\( \beta \)-gal, or PBS for 48 hours. Cells were washed with ice-cold PBS with phosphatase inhibitors. Nuclear proteins were extracted using the Nuclear Extract Kit (Active Motif) according to the manufacturer’s instruction. NF-κBp65 or SP1 transcription factor activity was quantitatively detected by TransAM NF-κBp65 or SP1 kit (Active Motif). Nuclear extract (20 \( \mu \)g) was used for each reaction. The specific primary antibody and the secondary antibody conjugated to horseradish peroxidase were incubated with the nuclear extracts following the vendor’s manual. The colorimetric reading (at 450 nm) was determined in Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Each experiment was done thrice.

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**References**


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