ABSTRACT

Expression of Fas (CD95, APO-1), a cell surface receptor capable of inducing ligand-mediated apoptosis, is involved in tissue homeostasis and elimination of targeted cells by natural killer and T cells. Corruption of this pathway, such as reduced Fas expression, can allow tumor cells to escape elimination and promote metastatic potential. In this study, the status of Fas expression has been examined in the parental SAOS human osteosarcoma cells that do not metastasize and in selected variants that cause lung metastases in 16 weeks (LM2) or 8 weeks (LM6) after i.v. injection into nude mice. Fas expression correlated with the metastatic potentials of the three cell lines. Northern and fluorescence-activated cell-sorting analyses indicated that LM6 cells expressed Fas at a lower level than seen in the parental cells. Infection of the LM6 cells with an adenoviral vector containing the murine interleukin (IL)-12 gene (Ad.mIL-12) or treatment with recombinant murine IL-12 resulted in a dose-dependent up-regulation of Fas. The up-regulation of Fas by IL-12 was also demonstrated in human etoposide-resistant MDA-MB-231 breast cancer cells. [3H]Thymidine growth inhibition studies indicated that the cell surface Fas induced after IL-12 exposure was functional and able to mediate cell death on cross-linking with anti-Fas. We also demonstrate that this effect is independent of IFN-γ, whereas these cell lines are sensitive to IFN-γ. Incubation with IFN-γ does not increase susceptibility to Fas-mediated cell death, nor do these cells produce IFN-γ with or without IL-12 treatment. We hypothesize that expression of Fas may play a role in the elimination of metastatic tumor cells in the lung, an organ in which Fas ligand is expressed. The antitumor activity of IL-12 may be secondary in part to its ability to up-regulate Fas expression on tumor cells, which subsequently increases immune-mediated destruction of osteosarcoma cells.

INTRODUCTION

At presentation, the majority of osteosarcoma patients have pulmonary micrometastases, and 30–40% of these patients will relapse with pulmonary metastases despite aggressive chemotherapy and surgical resection of the primary tumor (1–3). Neither surgery nor salvage chemotherapy has significantly improved the metastasis-free survival rate (4, 5). Because of the poor response rate of patients with relapsed osteosarcoma, we previously developed an experimental mouse model to assess the efficacy of new therapeutic agents against pulmonary metastases (6). Using this model, we have demonstrated that nasal delivery of an adenoviral vector containing the mIL-12 gene resulted in the inhibition of pulmonary metastases (7).

The cytokine IL-12 is a key component in numerous immune functions including stimulation of T cells and NK cells and regulation of several cell adhesion molecules (8, 9). It promotes the production of IFN-γ by T and NK cells. IL-12 is a heterodimer composed of p35 and p40 subunits. The smaller subunit is required for signaling through the IL-12R, whereas the larger subunit facilitates protein binding (10). The demonstration of significant antitumor activity in several preclinical animal tumor models has piqued interest in the therapeutic use of IL-12 (11–13). The mechanisms underlying this antitumor activity are incompletely understood but may be related to the ability of IL-12 to inhibit angiogenesis (14) or stimulate T cells and NK cells (15).

In this report, we document that IL-12 directly up-regulated the expression of Fas (CD95, APO-1), a cell surface molecule capable of inducing ligand-mediated apoptosis (16), on human osteosarcoma and breast cancer cells. This up-regulation occurred in response to either the addition of the recombinant molecule or transfer of the IL-12 gene. Whereas in immune cells, IL-12 induces IFN-γ production, and IFN-γ can up-regulate Fas (17, 18), we report here, for the first time, the ability of IL-12 to directly influence Fas expression, independent of IFN-γ, in these cell lines. Fas up-regulation was specific for IL-12 and could not be duplicated by the transfer of either β-gal or topol genes, which serve as controls for any effect due to the adenoviral vector. Fas-mediated apoptosis has been implicated as a potential regulator of tumor development, outgrowth, and metastasis (19–22). We hypothesize that, in addition to its antiangiogenic activity and its ability to activate T and NK cells, the ability of IL-12 to increase Fas surface expression may contribute to its antitumor activity.

MATERIALS AND METHODS

Cell Lines. The human osteosarcoma cell line SAOS-2 and the T-lymphoma cell line Jurkat-E6 were obtained from the American Type Culture Collection (Manassas, VA). The LM6 and LM2 cell sublines were derived from SAOS-2 cells by selection in 0.9% agarose followed by repeated i.v. cycling through the lungs of nude mice (6). Pulmonary metastases are evident 8 weeks (LM6) or 16 weeks (LM2) after i.v. injection. Parental SAOS-2 cells do not form metastases. The SAOS cell lines were maintained in Eagle’s MEM. MDA-VP, derived from human MDA-MB-231 breast cancer cells, was maintained in DMEM and 1 μM etoposide (23). Both media were supplemented as described previously (23). The cells were tested routinely and found to be free of Mycoplasma contamination (M. A. Bioproducts, Walkersville, MD). The Jurkat-E6 cell line was cultured in complete medium (RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine) at 5 × 104 cells/ml.

Northern Blot Analysis. Total RNA was isolated by using Trizol reagent (Life Technologies, Inc., Grand Island, NY), electrophoresed on 1% formaldehyde/agarose, and transferred to Hybond-N+ membrane (Amersham, Arlington Heights, IL). mIL-12 [created from the pCAGGSIL-12 plasmid; provided by Dr. K. Okuda (Okayama University School of Medicine, Okayama, Japan)], human IL-12 [Dr. Xaio Jing Ma (University of Pennsylvania, Philadelphia, PA)], Fas, and glyceraldehyde-3-phosphate dehydrogenase probes were labeled by using the Rediprime DNA labeling system (Amersham).
RT-PCR. Total RNA was isolated from cells in culture at various times by using Trizol reagent (Life Technologies, Inc.). RT-PCR detection of IL-12R expression was performed as described using primers specific for human the IL-12R b1 subunit (24). Primers for b2-microglobulin were used as a control. RNA from Jurkat-E6 cells was used as a control. RT-PCR was performed with 5 μg of total RNA by using SuperScriptr II reverse transcriptase (Life Technologies, Inc.). The product was then used in PCR amplification with Taq-DNA polymerase (Roche, Indianapolis, IN).

Fluorescence-activated Cell-sorting Staining and Flow Cytometry. Indirect staining and flow cytometric analyses were carried out as described previously (25). Cells (5 X 10^6) were plated in 6-well plates, incubated for the appropriate periods, collected, and incubated with either 1 μg of R-PE-conjugated mouse antihuman Fas antibody (clone DX2; Pharmingen, San Diego, CA) or 1 μg of isotype-matched, PE-conjugated, control mouse anti-human IgG1 antibodies (Sigma Chemical Co., St. Louis, MO). Samples were analyzed with a FACSscan (Becton Dickinson, Mountain View, CA).

Cytostasis Assay. Cytostasis was quantified by measuring [3H]thymidine incorporation, as described previously (26). Cell death was initiated by incubating the cells with an anti-human, anti-Fas antibody (clone CH11) that induces signaling by trimerizing Fas molecules on the cell surface. Briefly, 5 X 10^6 LM6 cells were plated in each well of a 96-well plate, incubated with medium alone or medium containing 1 X 10^3 pfu/cell Ad.mIL-12. Twenty μl of either medium alone, medium containing 1 ng/ml Fas antibody (clone CH11; Medical and Biological Laboratories, Nagoya, Japan), or medium containing 1 ng/ml mouse IgM (Pharmingen) were added to each well, and the plates were incubated for 60 h. The cultures were labeled with 0.2 μCi/well [3H]thymidine during the last 24 h. The cells were then washed twice with HBSS and lysed with 0.1 ml of 0.1 N KOH. Radioactive incorporation was quantified, and cytostasis was calculated as described.

Cytotoxicity Assay. Assays were performed as described previously (23). Cells were plated on a 96-well plate and fed with either medium alone or medium containing 300 pg/ml rmIL-12. After incubation for 10 h, 100 μl of either medium alone, medium containing 1 ng/ml Fas antibody (clone CH11), or medium containing 1 ng/ml mouse IgM (Pharmingen) were added to each well, and the plates were incubated for 24 h. Forty μl of 0.42 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well, and the cells were incubated for 2.5 h. Medium was then aspirated, and cells were lysed with 100 μl of DMSO. Cytotoxicity was quantified by using a 96-well microtiter plate reader at 570 nm.

Reagents and Adenoviral Vectors. A bicistronic adenoviral vector (Ad.mIL-12) carrying the cDNA of both the p35 and p40 subunits of mIL-12 (23), were propagated in 293 cells and purified as described previously (23).

RESULTS

Effect of IL-12 Gene Transfer on Fas Expression. LM6 cells expressed significantly less Fas mRNA and cell surface Fas than did the parental cells and the LM2 subline (Fig. 1, A and B). Additionally, LM6 cells express the b1 subunit of the IL-12R (Fig. 1C). Although the primers (nucleotides 1073–2239) that successfully indicate the b1 subunit in Jurkat-E6 cells failed to do so in LM6 cells, an alternate primer set (nucleotides 239–807) did detect the b1 transcript in the tumor cells. A similar expression pattern was found by Grohmann et al. (24) in Concanavalin A lymphoblasts and dendritic cells. Exposure of LM6 cells to Ad.mIL-12 (1000 pfu/cell) for 48 h results in the production of IL-12 in concentrations greater than 1000 pg/ml (7), and Northern analysis demonstrated a dose-dependent increase in both mIL-12 expression and Fas expression (Fig. 2A). LM6 cells infected with 1000 pfu/cell Ad.mIL-12 demonstrated a 6.3-fold increase in Fas as compared with Fas expression in untreated control cells. In contrast, Fas expression did not increase after infection with 1000 pfu/cell Ad.b-β-gal. The maximum effect on Fas took place 48 h after infection (data not shown). Fluorescence-activated cell-sorting analysis determined that 40% of the Ad.mIL-12-treated LM6 cells were Fas positive compared with 17% of the Ad.b-β-gal-treated cells. Mean fluorescence intensity, a measurement of antigen density on the cell surface, also increased accordingly with treatment, from 4.63 to 6.88. Together, these data demonstrate that IL-12 increased both the number of Fas-positive cells and the density of the antigen on the cell surface.

Fig. 1. Fas expression in SAOS-2 parental cells and sublines. A, total RNA was isolated from 2 X 10^6 cells, and Northern blot analysis was performed with a human Fas cDNA probe. B, cells (2.5 X 10^6) were stained with biotinylated antibodies against Fas, treated with streptavidin-PE, and analyzed by flow cytometry. C, total RNA was isolated from 2 X 10^6 cells, and RT-PCR was performed with primers specific for the human IL-12R b1 subunit, comprised of the indicated nucleotides, with primers for β2-microglobulin as a control for loading.

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This up-regulation of Fas by IL-12 was not unique to LM6 osteosarcoma cells. MDA-VP cells are etoposide-resistant breast cancer cells that express less Fas than their parental MDA-MB-231 cells (Fig. 2B, Lanes 1 and 2). Exposure to Ad.mIL-12 (Fig. 2B, Lane 3), but not to Ad.β-gal (Fig. 2B, Lane 4) or an adenoviral vector containing the htopoIIa gene (Fig. 2B, Lane 5), also increased the expression of Fas on MDA-VP cells.

Effect of rmIL-12 on Fas Expression. Treatment of LM6 cells with rmIL-12 also increased Fas expression. After a 7-h incubation with 300 pg/ml rmIL-12 (Fig. 3), 66% of LM6 cells were Fas positive compared with 28% in the untreated samples. Fas expression peaked 8–10 h after treatment and returned to baseline levels by 21 h. Similar results were found with the administration of recombinant human IL-12.

Biologically Active IL-12 Is Required for Induction of Fas. The biological activity of IL-12 requires a heterodimeric structure consisting of both p35 and p40 subunits, whereas the p40 homodimer has no biological activity (11). To determine whether both subunits were required for Fas up-regulation, we transfected LM6 cells with either the pcAGGSIL-12 plasmid containing genes for both subunits or the plasmid containing only the p40 subunit of IL-12 (7). Clones were selected, isolated, and tested for IL-12 expression (Fig. 4A). Increased cell surface Fas was demonstrated in clone b expressing both IL-12 subunits, but not in clone c expressing only the p40 subunit or in clone a expressing neither subunit (Fig. 4B).

Assaying Functionality of IL-12-induced Fas. Parental SAOS-2 cells were treated with 1 ng/ml Fas-mediated cell death-inducing antibody (CH11), resulting in 19% growth inhibition as measured by [3H]thymidine incorporation cytostasis assay (Fig. 5). LM6 cells treated with CH11 had no significant growth inhibition compared with untreated cultures. Additionally, LM6 cells treated with CH11 and Ad.β-gal had no significant growth inhibition compared with cells treated with Ad.β-gal alone. However, infection of the LM6 cells with Ad.mIL-12 (1×10^3 pfu/cell) for 48 h with 1 ng/ml CH11 resulted in

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Fig. 5. Effect of IL-12 on Fas-mediated cell death. A total of 5 × 10⁴ parental cells (P), LM6 cells stably transfected with the IL-12 gene (clone b), and unmodified LM6 cells (LM6) were treated for 60 h as indicated with cell death-inducing anti-Fas antibody (CH11), Ad.β-gal, Ad.mIL-12, or an IgM isotype control. Growth inhibition was assayed by using [³H]thymidine incorporation.

~25% cytostasis over the levels due to the cytotoxic effect of the adenosine. Also, LM6-IL-12 transfectant clone b had an average of 30% cytostasis when incubated with CH11. Similarly, incubation of LM6 cells with rmIL-12 (300 pg/ml) for 10 h followed by a 1–24-h treatment with CH11 resulted in 73–92% cytotoxicity as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, compared with 5% when cells were treated with CH11 or IL-12 alone (data not shown). These findings indicate that the cell surface Fas induced after IL-12 exposure was functional and able to mediate cell death.

Role of IFN-γ. Because in immune cells IL-12 induces IFN-γ production, and IFN-γ can up-regulate Fas (17, 18), we investigated the role of IFN-γ in IL-12 induction of Fas on LM6 cells. Cultured supernatants from LM6 cells treated for 10 h with 300 pg/ml rmIL-12 or for 48 h with Ad.mIL-12 contained no detectable hIFN-γ as measured by ELISA (lower detection limit, 3.906 pg/ml). Furthermore, Fas levels did not change significantly with either 200 or 2000 units/ml rhIFN-γ treatment, whereas in PBLs, 200 units/ml rhIFN-γ increased Fas levels by 25% (data not shown). Whereas hIFN-γ-neutralizing antibody (1–100 μg/ml) prevented the IFN-γ-induced up-regulation of Fas in PBLs, it had no effect on IL-12 induction of Fas on LM6 cells infected with Ad.mIL-12 (data not shown). Additionally, SAOS cells are exquisitely sensitive to IFN-γ, with an IC₅₀ at 48 h of 1000 units/ml (26). We find a similar sensitivity in LM6 cells, and we do not observe an increase in cell death after incubation with rhIFN-γ and CH11 beyond that due to the cytotoxic effect of the cytokine (data not shown). Furthermore, neither LM6 cells exposed to IL-12 nor LM6 cells transfected with the IL-12 gene exhibit the gross morphological changes indicative of cell death or significant growth inhibition, as measured by [³H]thymidine assays, as is seen with IFN-γ treatment. These data indicate that in this in vitro system, the induction of Fas expression after IL-12 exposure occurs independently of IFN-γ.

DISCUSSION

The data presented here demonstrate that IL-12 up-regulated Fas mRNA and cell surface protein expression in LM6 and MDA-VP cells. This up-regulation was achieved either by incubating the cells with recombinant IL-12 (murine or human) or by transfer of the mIL-12 gene by two different methods. We elected to use the mIL-12 gene to subsequently test the consequences of gene transfer on host response in a mouse model. Whereas human cells respond to mIL-12, murine cells do not respond to human IL-12. Therefore, testing the effect of gene transfer in a mouse model necessitates using the murine gene. Cells transfected with the mIL-12 gene or exposed to an adenoviral vector containing the mIL-12 gene showed production of Fas mRNA and protein concurrent with the up-regulation of IL-12. Fas did not increase in cells transfected with only the p40 subunit, indicating that biologically active IL-12 was required for this up-regulation. Furthermore, the Fas induced by IL-12 was functional, as demonstrated by cell death on cross-linking with the anti-Fas antibody CH11. Although IFN-γ is known to up-regulate Fas expression, and IL-12 stimulates IFN-γ production in immune cells (17, 18), we were unable to demonstrate the production of IFN-γ by LM6 cells in response to IL-12. Furthermore, treatment of these cells with IFN-γ did not increase Fas expression, and treatment with IFN-γ-neutralizing antibody did not suppress the induction of Fas by IL-12. There is no increase in cell death beyond that due to the cytotoxic effects of IFN-γ when CH11 is added to LM6 cells incubated in IFN-γ. This is as we expected because LM6 cells do not express high amounts of Fas, through which CH11 could trigger cell death. These data strongly suggest that the increased Fas expression seen in response to IL-12 is independent of IFN-γ.

The up-regulation of Fas was not unique to osteosarcoma cells. Increased Fas expression was also seen in MDA-VP breast cancer cells after exposure to Ad.mIL-12. Furthermore, transfer of the genes for β-gal or human topoII gene, the latter of which enhances the cellular sensitivity of MDA-VP cells to etoposide (23), did not alter Fas expression, indicating that the enhanced Fas expression was specific for IL-12.

In 1998, Grohmann et al. (24) characterized a novel IL-12R found on dendritic cells. In contrast to the receptor found on T and NK cells, which induces a signal through Janus-activated kinase/STAT molecules, this receptor instead prompts the nuclear localization of NF-κB family members. Chan et al. (36) have shown that NF-κB activation was required for Fas up-regulation. Using the same fos promoter-driven luciferase reporter gene constructs as described in Chan et al. (36), we have preliminary data that demonstrate that Ad.mIL-12 up-regulates Fas on a transcriptional level. These results, when combined with our own, suggest to us a pathway by which IL-12 is able to directly regulate Fas expression and suggest that this effect may not exist in lymphocytes.

Although the IL-12R has rarely been found on nonlymphoid tissues, receptors to cytokines thought to be involved only in immune functions can be found on a number of solid tumors (29). Receptors for another proinflammatory cytokine, IL-17, can be found on osteogenic cells from which osteosarcoma is derived (30).

Expression of both the β₁ and β₂ subunits of the IL-12R is necessary for high-affinity binding of IL-12 (31, 32). The β₁ subunit is required for IL-12 responsiveness, and binding of this subunit induces a pathway that results in the induction of a variety of events including cell proliferation and cytokine production of immune cells (33–35). We have shown that the LM6 cells, which respond to IL-12 by up-regulating Fas, possess the IL-12R β₁ subunit. Whereas published primers specific for the β₂ subunit have failed to demonstrate the presence of that subunit, it is possible that the primers we have tried thus far are inappropriate for the IL-12R found on these tumor cells. Indeed, we found that primer selection was crucial for identifying the IL-12R β₁ subunit (Fig. 1C). Primers consisting of nucleotides 1073–2239 failed to demonstrate the presence of the β₁ subunit, whereas those primers specific for nucleotides 239–807 were successful in LM6 cells.

Chan et al. (36) have shown that NF-κB is involved in the up-
regulation of Fas in Jurkat-E6 cells. In T cells, exposure to IL-12 activates the STAT4 transcription factor (37). However, Grohmann et al. (24) found that IL-12 treatment of dendritic cells failed to involve the STAT family and instead resulted in the nuclear localization of NF-κB family members. Indeed, PCR primers specific for the IL-12R β1 expressed in dendritic cells also yielded positive results in the SAOS cell line. We therefore speculate that osteosarcoma cells possess an IL-12R that activates NF-κB, which can then up-regulate Fas expression. Investigations are under way to determine whether NF-κB is indeed involved in the up-regulation of Fas by IL-12 in these cells.

The ability of tumor cells to modulate the Fas system would be a distinct advantage for cell types like osteosarcoma that commonly metastasize to the lung. Because resident lung cells express the Fas ligand on their surfaces, the lung is considered an “immune privileged” site, an area in which inflammatory responses are controlled though the eradication of antigen-specific cytotoxic T cells that invade the tissue (12). This control is accomplished through the binding of Fas ligand on resident cells to the receptors of the T cells, which induces apoptosis in the T cells before they can instigate an immune response. Tumor cells that have either an ineffective Fas pathway or down-regulated Fas cell surface expression can survive the assault by cytotoxic T cells or by resident cells in an immune privileged site. Tumor cells that possess intact Fas pathways with significant Fas cell surface expression may have difficulty thriving in the lung, where apoptosis can be induced by the Fas ligand. Indeed, both parental and LM2 cells, which express high levels of Fas, form few if any lung metastases after i.v. injection. By contrast, injection of LM6 cells, which normally express low levels of Fas, produces metastases within 8 weeks (6). Exposing LM6 cells to IL-12 up-regulates Fas expression, which in turn could increase the susceptibility of the cells to ligand-induced apoptosis by the resident lung cells. We have demonstrated that LM6 clones transfected with the IL-12 gene have lesser tumorigenicity and that intranasal delivery of Ad.ML-12 induced regression of LM6 lung metastases (7).

IL-12 demonstrated significant antitumor activity in several pre-clinical models that include both gene therapy and recombinant IL-12 (11, 15). The mechanisms underlying IL-12-mediated tumor regression are not completely understood but may involve activation of TNF and NK cells or antiangiogenic properties in the overall tumor rejection process. We propose that the up-regulation of Fas on tumor cells by IL-12 may influence elimination of the cells via Fas/Fas ligand-mediated apoptosis. 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 use (38). 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 may aid in strategies to use this cytokine more effectively while avoiding toxic complications. Local gene delivery, for example, may limit systemic exposure while still supplying a high concentration to the tumor area.

In summary, IL-12 is a potent stimulant of the immune response. It can be an effective weapon against cancer, but it can also cause toxic complications that interfere with its use. Understanding the mechanisms of IL-12 action is important for our ability to exploit the use of this agent clinically. In this report, we have illustrated one additional activity of IL-12, namely, its ability to up-regulate Fas expression, a potent death receptor on tumor and normal cells.

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Interleukin (IL)-12 and IL-12 Gene Transfer Up-Regulate Fas Expression in Human Osteosarcoma and Breast Cancer Cells

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