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Fas Expression in Metastatic Osteosarcoma Cells Is Not Regulated by CpG Island Methylation

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Abstract

Fas expression in osteosarcoma (OS) cells is inversely correlated with the metastatic potential of OS to the lung. The purpose of this study was to determine whether loss of Fas expression in metastatic OS cells is secondary to DNA methylation of CpG islands in the Fas gene. SAOS-2 cells have high levels of Fas expression and do not form lung metastases when injected intravenously, whereas LM7 cells have low levels of Fas expression and do produce lung metastases. Using the endonucleases *HpaII* and *MspI* and a polymerase chain reaction-based methylation assay, we found that all four CpG sites in the CCGG sequence in the Fas promoter region were unmethylated in both SAOS-2 and LM7 cells. We performed detailed analysis of the 28 and 46 CpG sites in the Fas promoter and first intron region, respectively, using bisulfite-modified genomic DNA sequencing. More than 99.8% of the examined CpG sites were unmethylated and there was no difference of CpG methylation in SAOS-2 and LM7 cells as well as LM7 metastatic lung tumor tissue samples. Treatment of LM7 cells and another OS cell line, DLM8 with low levels of Fas expression, with demethylation agent, 5-azadeoxycytidine (AzadC), did not change the Fas expression and did not increase sensitivity of AzadC-treated cells to Fas ligand (FasL) treatment. In conclusion, our data indicate that decreased Fas expression in OS cells is not secondary to DNA methylation of CpG islands in the Fas gene and that Fas expression cannot be increased by using demethylation agents.

Keywords

Fas gene; Osteosarcoma; CpG methylation

INTRODUCTION

Osteosarcoma (OS), the most common primary malignant bone tumor, has a propensity for metastasis to the lung. Patients with OS lung metastases have poor prognoses and limited therapeutic options (1). Despite the use of aggressive combination chemotherapy and surgery, the disease-free and long-term survival rates in patients with OS have remained unchanged for more than 20 years. Understanding the molecular mechanisms that contribute to the ability of OS cells to metastasize to the lung is essential for developing new therapeutic strategies for this disease.

Fas (a member of the tumor necrosis factor receptor family) is expressed in a variety of normal and neoplastic cells, including OS cells (2–6). Interaction of Fas with its ligand (FasL) induces apoptosis. Fas-mediated apoptosis has been associated with tumor regression and thus plays an important role in tumor development and progression. FasL is constitutively expressed in lung epithelial cells. Therefore, Fas⁺ cells can be eliminated from the lung upon interaction

with FasL. Indeed, we previously showed that Fas plays an important role in the ability of OS cells to metastasize to the lung (2,3). Specifically, Fas expression was inversely correlated with the lung metastatic potential using two different mouse models (3,7). OS cells with high levels of Fas expression were eliminated from the lung following IV injection, whereas cells with low levels of Fas expression escaped apoptosis, resulting in the development of OS lung metastases (2,3,7). Fas⁺ cells were able to form lung metastases in FasL-deficient gld mice (7).

The Fas/FasL pathway has been identified as a key mediator of chemotherapy-induced apoptosis in leukemia and several solid tumors (4,5,8). Therefore, alterations in Fas expression may also impact the sensitivity of OS cells to chemotherapy-based regimens. Downregulation of Fas expression may be a mechanism by which OS cells can circumvent FasL-mediated cell death in addition to chemotherapy-induced cell death. To date, however, the mechanism underlying the regulation of Fas expression in OS cells is poorly understood.

DNA methylation of promoter-associated CpG islands results in transcriptional silencing of genes. This process contributes to the alteration of gene expression in cancer cells (9). For example, in previous studies, DNA methylation of CpG islands was responsible for silencing of the Fas gene in antigen-specific cytotoxic T cells and in prostate and some colon cancer cells but did not account for low levels of Fas expression in ovarian cancer cells (9–13). In the present study, we sought to determine whether loss of Fas expression in metastatic OS cells is secondary to DNA methylation of CpG islands in either the promoter or first intron region of the Fas gene. Our data demonstrated that decreased Fas expression is most likely not secondary to DNA methylation. Almost all of the CpG sites in both the Fas promoter and first intron region in metastatic OS cells were unmethylated. Also, the number of methylated CpG sites did not correlate with Fas expression levels. These data were further confirmed when OS tumor cells were treated with AzadC demethylation agent. AzadC did not increase the expression of Fas or cell sensitivity to the FasL.

MATERIALS AND METHODS

Reagents

Eagle's modified essential medium, nonessential amino acids, sodium pyruvate, minimal essential medium-vitamins, L-glutamine, and 2.5% trypsin were purchased from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was purchased from Intergen (Purchase, NJ). *MspI* methylase, *HpaII* methylase, and the restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except where indicated. Primer oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX). 5-Aza-2'-deoxycytidine (AzadC) was purchased from Sigma.

Cell Lines and Tumor Tissue Samples

SAOS-2 human parental nonmetastatic OS cells were obtained from the American Type Culture Collection (Manassas, VA). The metastatic LM7 cell line was developed by repetitive cycling of SAOS-2 cells through the lungs of nude mice seven times (14). These cells were cultured in Eagle's modified essential medium supplemented with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 2× minimal essential medium vitamin solution, and 10% heat-inactivated (56°C for 30 min) fetal bovine serum. A monolayer culture of cells was maintained at 37°C in a humidified 5% CO₂ incubator. LM7 metastatic lung tumor samples were excised from nude mice 6 weeks after IV injection of 10⁶ LM7 cells.

Dunn LM8 (DLM8) cell line was obtained from Dr. Akira Myoui (University of Osaka, Osaka, Japan) and maintained in Dulbecco's modified essential medium with the same supplements as describe for SAOS-2 and LM7 cells.

Flow Cytometric Analysis

SAOS-2 and LM7 Cells (1×10^6) were plated in six-well plates overnight. Cells were then collected and incubated with 1 μ g of an r-phycoerythrin-conjugated mouse anti-human Fas or hamster anti-Fas antibody (Pharmingen, San Diego, CA) and analyzed using a FACScan (Becton Dickinson, Mountain View, CA) as described previously (15).

Polymerase Chain Reaction-Based Methylation Assay

The *MspI* and *HpaII* restriction endonucleases were applied in a polymerase chain reaction (PCR)-based methylation assay as described previously with modifications (16). Both *MspI* and *HpaII* recognize the CCGG sequence, which contains a CpG site. *HpaII* cleaves only the CCGG sequence (unmethylated cytosines), whereas *MspI* cleaves CCGG and C^mCGG (methylated internal cytosine). Thus, the combination of *MspI* and *HpaII* can be used to analyze the methylation status of CpG in the CCGG sequence. DNA samples (0.2 μ g each) were incubated with 20 units of *MspI* and *HpaII* at 37°C for 3 h in a total 20- μ l reaction volume followed by inactivation of the enzymes by heating at 65°C for 15 min. DNA samples pretreated with *HpaII* or *MspI* methylase to methylate internal cytosines and external cytosines, respectively, before *HpaII* and *MspI* digestion, were used as positive controls for DNA methylation. Each sample (0.5 μ g of DNA) was incubated with 8 units of either of the methylases in a total 10- μ l reaction volume at 37°C for 1 h and inactivated at 65°C for 15 min; *MspI* or *HpaII* digestion was then performed.

PCR was performed to amplify the Fas DNA fragment (−564 to −18) that included four CpG sites of CCGG sequences. Digested DNA samples (4 μ l each) were used as templates and specific primers as follows: 5'-AATTAGCCAAGGCTCCTGTACC-3' and 5'-GCA TGGTTGTTGAGCAATCCTC-3'. The PCR conditions were 3 min at 94°C; 35 cycles of 1 min at 94°C, 30 s at 56°C, and 1 min at 72°C; and an extension step of 5 min at 72°C. The PCR products were subjected to electrophoresis on a 2% agarose gel with ethidium bromide and visualized under ultraviolet light.

Bisulfite Genomic Sequencing

Genomic DNA was isolated from cultured OS cells and xenograft tumor tissues using the DNeasy DNA isolation kit (Qiagen, Valencia, CA) followed by *EcoRI* digestion. Bisulfite modification of genomic DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Orange, CA) following the manufacturer's directions. Briefly, 1 μ g of purified DNA (20 μ l) was incubated in 130 μ l of CT Conversion Reagent at 98°C for 10 min and 64°C for 2.5 h, resulting in conversion of unmethylated cytosines into uracil. After DNA purification, the bisulfite-modified DNA was amplified using two rounds of PCR with nested primers. The primers used to amplify the promoter region of the Fas gene (containing 28 CpG sites) were described previously (9); the primers used to amplify the first intron region of the Fas gene (containing 46 CpG sites) were designed using the online program MethPrimer (<http://www.urogene.org/methprimer/>): first-round PCR primers, 5'-GGAGGATTGTTTAATAATTATGTTGG-3' and 5'-ATAAACAACCTCCAAAACTCATT-3'; second-round PCR primers, 5'-TGGATTTTTTTATTTTTGTTGAGTT-3' and 5'-TCTAAAACTACAACTCTCTCCCC-3'.

The PCR conditions were as follows: 3 min at 94°C; 35 cycles of 1 min at 94°C, 30 s at 56°C, and 1 min at 72°C; and an extension step of 5 min at 72°C. Second-round PCR was performed

using 1 µl of the first-round PCR product in a total volume of 50 µl. The PCR products were purified using electrophoresis on 1% agarose gels (QIAquick gel extraction kit; Qiagen), recovered using ethanol precipitation, and then cloned using ligation into pCR 2.1-TOPO cloning vectors with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) followed by introduction into TOP10 One Shot competent bacteria (Invitrogen). Plasmid DNAs isolated from at least five clones for each PCR reaction product were subjected to DNA sequence analysis at The University of Texas M. D. Anderson Cancer Center DNA Analysis Core Facility or by SeqWright DNA Technology Services (Houston, TX). As compared with the original genomic Fas DNA sequence, the methylation of CpG was determined as appearing unconverted cytosines in the CpG sites in bi-sulfite-modified DNA.

Cytotoxicity Assay

The sensitivity of OS cells to AzacC or soluble FasL (sFasL) (Alexis Biochemicals, San Diego, CA) was determined by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1,000 cells/well were grown in 96-well plates and treated with AzadC for 24 h and followed by 100 ng/ml sFasL for another 24 h; wells with nontreated cells served as positive controls and wells without cells were used as negative controls. MTT reagent was added to each well at a concentration of 0.08 mg/ml for 2–4 h. Cells were lysed with 0.1 ml of dimethyl sulfoxide. Cytotoxicity was quantified by using a microtiter plate reader at 570 nm.

Genomic DNA Digestion

Genomic DNA obtained from cultured LM7 cells was digested with *MspI* and *HpaII*. The global methylation pattern of genomic DNA was analyzed as indicated by the sensitive of the DNA response to the restriction endonuclease digestion, which has been described previously (17).

RESULTS

Fas Expression in SAOS-2 and LM7 Cells

Fas expression was significantly lower in LM7 cells than in SAOS-2 cells (Fig. 1). While 82% of the SAOS-2 cells expressed Fas, less than 24% of the LM7 cells were Fas⁺.

DNA Methylation Status of CpG Islands in OS Cells

We assessed the DNA methylation status of CpG sites in the CCGG sequence using a PCR-based methylation assay. Sequence analysis showed four CpG sites in this sequence in the promoter region of the Fas gene. DNA samples extracted from both SAOS-2 and LM7 cells were unmethylated as demonstrated by the absence of a PCR product following digestion with either *MspI* or *HpaII* (Fig. 2, lanes 2 and 4). These data indicate that all four CpG sites in the CCGG sequences of both SAOS-2 and LM7 cells were unmethylated.

Using the MethPrimer program, we determined the number of CpG-enriched regions of the Fas gene (Fig. 3A). The promoter and first intron region of the Fas gene contained 28 and 46 CpG sites, respectively. We analyzed the methylation status of these CpG sites in the SAOS-2 and LM7 cells using bisulfite-modified genomic DNA sequencing (Fig. 3B). Only 0.01–0.02% of the CpG sites in the Fas promoter and first intron region were methylated in both SAOS-2 and LM7 cells. We found no difference in Fas DNA methylation in these two cell types. Similarly, in LM7 metastatic lung tumor tissue samples, less than 0.01% of the CpG sites in the promoter region of the Fas gene were methylated, and we found no CpG site methylation in the first intron region. These results indicate that the Fas gene was unmethylated in both the parental SAOS-2 and metastatic LM7 cells.

Inhibition of DNA Methylation Did Not Change the Expression of Fas in OS Tumor Cells or Their Sensitivity to FasL

To further confirm our findings that DNA methylation is not responsible for the changes in the expression of Fas receptor in OS tumor cells, we treated two different OS cell lines, LM7 and DLM8, with AzadC an irreversible inhibitor of DNA methylation. AzadC treatment indeed resulted in a decrease of global genomic DNA methylation in LM7 cells; however, flow cytometry showed that Fas expression was unchanged in these cells after treatment with AzadC (Fig. 4). In accordance with this, the sensitivity of LM7 and DLM8 cells to FasL was not enhanced by AzadC (Fig. 5). These data indicate that AzadC did not increase the expression of Fas in OS tumor cells or sensitize them to Fas-mediated apoptosis.

DISCUSSION

We have demonstrated that Fas expression in parental nonmetastatic SAOS-2 cells is higher than that in metastatic LM7 cells (18). We previously demonstrated that LM7 lung metastases are Fas⁻ (19). In the present study, we hypothesized that DNA methylation of CpG islands is responsible for the downregulation of Fas expression in metastatic OS cells. However, our data demonstrated no difference in the DNA methylation status of the Fas gene between the parental and metastatic subline. Indeed, the CpG sites in the promoter and first intron of the Fas gene were unmethylated in both SAOS-2 and LM7 cells as well as in LM7 metastatic lung tumor tissue samples.

Our laboratory has shown that the level of Fas expression in OS cells is inversely correlated with the cells' ability to metastasize to the lung. We also found that aerosol delivery of the interleukin-12 gene or gemcitabine and 9-nitrocamptothecin chemotherapy resulted in upregulation of Fas gene expression in established OS lung metastases with subsequent tumor regression (19–21). Other investigators have also reported that in addition to chemotherapy, interferon- γ , ultraviolet irradiation, viral infection, and wild-type p53 can induce Fas gene expression (22). Therefore, regulation of Fas expression may impact the sensitivity of tumor cells to certain therapies in addition to being critical for the metastatic potential of OS cells. Downregulation of Fas expression may be one mechanism by which tumor cells circumvent therapy-induced apoptosis. Defining the mechanism of Fas gene suppression may identify novel therapeutic strategies for the treatment of OS and other Fas⁻ tumors. DNA methylation is an epigenetic regulation mechanism involved in transcriptional silencing of gene expression. The Fas gene promoter (28 CpGs between sequences -590 and -1) and first intron region (46 CpGs between sequences 47 and 508) have relatively high CpG dinucleotide contents, which mean they can be subjected to DNA methylation. Authors have reported silencing of Fas gene expression controlled by DNA methylation in the Fas promoter region in some colon cancer cell lines (9). However, the DNA methylation status in colon cancers is controversial. Butler et al. (13) reported that the Fas gene was unmethylated in tumor samples obtained from patients with colorectal cancer.

In our study, we found no DNA methylation of CpG sites in CCGG sequences in the Fas promoter region as assessed using a PCR-based methylation assay. We further assessed the methylation status of all of the CpG sites in the Fas promoter and first intron region using bisulfite-modified DNA sequencing. Our data showed that more than 99.8% of the CpG sites were unmethylated in both SAOS-2 and LM7 cells and in LM7 meta-static lung tumor tissue samples. These results indicate that DNA methylation is probably not the mechanism that regulates the different levels of Fas expression in metastatic and nonmetastatic OS cells. This was further confirmed in our functional experiments, in which AzadC, an inhibitor of DNA methylation, was not able to increase expression of Fas on OS tumor cell surface. Because AzadC can inhibit DNA methylation of other genes and alter their expression we further demonstrated whether it can modify the sensitivity of OS cells to Fas-mediated apoptosis.

Treatment of OS cells with AzadC did not enhance their sensitivity to FasL, indicating that the expression of other downstream Fas signaling molecules expression was also not changed. Furthermore, AzadC did not increase the cells sensitivity to FasL.

Other mechanisms of gene regulation may control Fas gene expression in OS cells. Investigators have reported that nuclear factor (NF)- κ B is required for induction of the Fas promoter in response to phorbol myristate acetate and ionomycin in Jurkat cells (22). We previously showed that interleukin-12 but not interferon- γ upregulated Fas expression in human OS and Ewing's sarcoma cells by enhancing Fas promoter activity mediated by binding of NF- κ B to the κ B-Sp1 element (6). Therefore, an alternative hypothesis is that downregulation of Fas expression is mediated by a mechanism that interferes with NF- κ B binding to this κ B-Sp1 element. In summary, almost all of the CpG sites in both the promoter and first intron region of the Fas gene were unmethylated in SAOS-2 and LM7 cells. We observed no difference in the DNA methylation of the CpG islands in the two cell types. DNA methylation inhibition did not change the levels of Fas expression in two different OS cell lines and had no effect on their sensitivity to Fas-mediated apoptosis. We therefore conclude that the use of demethylation agents is unlikely to be beneficial for patients with OS lung metastasis and that other mechanisms of the Fas gene regulation should be investigated.

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Figure 1.

Fas expression in SAOS-2 parental nonmetastatic and LM7 metastatic OS cells. SAOS-2 and LM7 cells (1×10^6) were stained with an anti-human Fas antibody and analyzed using flow cytometry. The percentages of cells that expression Fas on the cell surface are indicated.

Figure 2.

Assessment of DNA methylation using a PCR-based methylation assay. Genomic DNA samples were extracted from SAOS-2 and LM7 cells. The samples were treated with *MspI* methylase (M, lane 1) or *HpaII* methylase (H, lane 3) or left untreated (lanes 2 and 4). Methylase-treated and untreated DNA samples were then incubated with *MspI* (lanes 1 and 2) or *HpaII* (lanes 3 and 4) and then subjected to PCR as described in Materials and Methods. Cleavage of the CCGG sequences by either *MspI* or *HpaII* resulted in loss of PCR products. The absence of PCR products in lanes 2 and 4 indicates that there was cleavage at the CCGG sequences; thus, these sites were unmethylated. The DNA samples treated with *HpaII* methylase or *MspI* methylase were used as positive controls for DNA methylation. PCR using H₂O (lane 5) served as the negative control. DNA without restriction (lane 6) was the positive control. Lane 7, marker.

Figure 3.

Assessment of DNA methylation using bisulfite-modified genomic sequencing. (A) Schematic representation of the CpG islands in the Fas promoter and first intron regions. Arrows indicate nested PCR primers. CpG sites (vertical lines) and CpG islands (enriched CpG sites, gray areas) are also indicated. (B) DNA methylation of CpG sites in SAOS-2 and LM7 cells and in LM7 metastatic lung tumors. Genomic DNA samples extracted from cultured cells or lung nodules were analyzed for methylation using bisulfite-modified genomic sequencing. Methylated CpG sites are indicated by filled circles and unmethylated sites are indicated by open circles.

Figure 4.

Aza-deoxycytidine (AzadC) treatment did not change expression of Fas receptor in osteosarcoma cells. (A) LM7 cells were not treated (shaded area) or treated with 0.1 mM (dashed black line) and 0.2 mM (solid black line) of AzadC for 48 h and followed by staining with PE-labeled anti-human Fas antibody. (B) DLM8 cells were not treated (shaded area) or treated with 2.0 μ M AzadC for 24 h (dashed black line) or 48 h (solid black line) and stained with PE-labeled anti-mouse Fas antibody. In both cases inhibition of DNA methylation did not change the levels of Fas expression in tumor cells. (C) LM7 cells were not treated or treated with 0.2 mM AzadC for 48 h. The genomic DNA was obtained and digested with restriction enzymes. More extensive DNA degradation in response to *Hpa*II digestion was observed in

LM7 cells treated with AzadC compared to not treated LM7 cells, indicating hypomethylation effect of AzadC treatment. Uncut genomic DNA (–) was used as a control.

Figure 5. Treatment of osteosarcoma cells with aza-deoxycytidine (AzadC) did not stimulate Fas-mediated apoptosis. (A) LM7 cells were pretreated with 0.1 mM of AzadC for 24 h and followed by treatment with 100 ng/ml FasL for another 24 h. Cell viability was determined by MTT assay as described in Materials and Methods. (B) DLM8 cells were pretreated with 2.0 μ M AzadC for 24 h and then with FasL as described for LM7 cells.