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Ganglioside GD2 as a Therapeutic Target for Antibody-Mediated Therapy in Osteosarcoma

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Abstract

BACKGROUND—Survival outcomes for patients with osteosarcoma have remained stagnant over the past three decades. Targeting of ganglioside GD2, a glycosphingolipid on the cell surface of some tumors, with immunotherapy has resulted in improved outcomes for patients with neuroblastoma. The expression pattern of GD2 was examined in osteosarcoma.

METHODS—Immunohistochemistry was performed on osteosarcoma samples from patients at the time of initial biopsy, definitive surgery, and recurrence. The intensity and location of staining were scored. Cell-based ELISA was performed on osteosarcoma cell lines to quantitate the level of GD2 expression.

RESULTS—Forty-four osteosarcoma samples were evaluated by immunohistochemistry, including 8 samples from the initial biopsy, 28 samples from the definitive surgery, and 8 samples from the time of recurrence. GD2 was expressed on all 44 osteosarcoma samples. Osteosarcoma tissue obtained at the time of recurrence showed higher intensity of staining compared to samples obtained at initial biopsy and definitive surgery ($p=0.016$). The majority of osteosarcoma cell lines expressed GD2 at higher levels than the neuroblastoma cell line BE(2)-C.

CONCLUSIONS—Ganglioside GD2 is highly expressed on osteosarcomas. Clinical trials are needed to assess the efficacy of targeting GD2 in patients with osteosarcoma.

Keywords

osteosarcoma; ganglioside GD2; immunotherapy; antibody

INTRODUCTION

Osteosarcoma is the most common primary bone tumor in childhood. Despite multiple clinical trials, cure rates for patients with osteosarcoma have not significantly improved over

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the past three decades, and survival for patients with metastatic or relapsed disease remains dismal.^{1,2} Novel approaches to the treatment of osteosarcoma are needed to improve outcomes for these patients.

Monoclonal antibodies targeted against cell surface antigens specific to tumor cells have been proven to be effective in patients with breast cancer, lymphoma, and neuroblastoma.³⁻⁵ Disialoganglioside GD2 is a glycosphingolipid that is expressed on the cell surface of limited normal adult tissue: the central nervous system, peripheral nerves, skin melanocytes, and mesenchymal stromal cells.⁶⁻⁸ GD2 is also expressed on tumor cells and has been shown to be uniformly expressed on the surface of neuroblastomas and many melanomas.^{6,9,10} Variable expression of GD2 has been shown in tumors in patients with lung cancer, central nervous system tumors, and sarcomas.^{11,12} Due to the relatively isolated expression of GD2 on malignant cells, it is an attractive target for antibody-mediated therapy and anti-GD2 antibodies have been shown to improve survival for patients with high-risk neuroblastoma^{5,13}

Expression of GD2 on the cell surface of osteosarcomas has not been fully evaluated, although prior reports suggest it is expressed¹¹. In this study we explore the utility of GD2 as a potential target for antibody-mediated therapy in patients with osteosarcoma.

MATERIALS AND METHODS

Patient Samples and Cell Culture

Human osteosarcoma tumor tissue was obtained from patients at the time of initial biopsy, definitive surgery, or at relapse. All tissue microarray samples were fixed in formalin and embedded in paraffin. Most samples were decalcified and the tissue microarray was constructed as previously described.^{14,15} All tumor samples used for ELISA were obtained from patients with osteosarcoma treated at Memorial Sloan Kettering Cancer Center (New York, NY). Tumor samples obtained at the time of definitive surgery all had 50% necrosis in order to be able to accurately assess GD2 expression across specimens. All specimen collection and analysis were performed in accordance with an Institutional Review Board approved protocol and all patients or their guardians provided written informed consent. Primary cell cultures were generated by standard collagenase disaggregation of tissues.¹⁶ All isolated cells were cultured in MEM supplemented with 20% FCS and antibiotics in a 5% CO₂ humidified atmosphere at 37°C. All short term cultures were established within 20 passages. The neuroblastoma cell line, BE(2)-C, and the fibrosarcoma cell line, HT1080, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in the suggested media and additives.

Immunohistochemistry

Slides that contained the paraffin-embedded microarray and control tissues were baked and subsequently deparaffinized. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol. Antigenic proteins were unmasked using universal antigen retrieval solution (IHC world, Woodstock, MD). The tissue was blocked with 10% normal goat serum in 5% bovine serum albumin (BSA) in TBS, and stained with 100 ug/ml 14.G2a antibody diluted in 5% BSA overnight. 14.G2a antibody was a generous gift from Dr. Karen Muszynski at the National Cancer Institute. Commercially available paraffin-embedded melanoma tissue known to uniformly express GD2 (BioChain Institute, Hayward, CA) was used as positive control, and the purified IgG2a diluted in the same diluent as above was used instead of the primary antibody as the negative control. Slides were placed in a humidified chamber during incubation with primary antibody. Detection of the antibody-binding reaction was carried out with biotinylated secondary antibody coupled with

streptavidin-horseradish peroxidase. Avidin-biotinylated enzyme complex (Vectastain ABC system, Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions. The tissue was treated with 3,3'-diaminobenzidine (Biofx Laboratories, Owings Mills, MD) to identify sites of additional antibody binding and counterstained with hematoxylin. The tissue was then dehydrated with alcohol, permeated with xylene, and mounted with Permount organic mounting solution (Fisher Scientific Inc., Pittsburgh, PA). Stained slides were viewed using a Nikon Inverted Microscope ECLIPSE TE200 attached to a CCD (Diagnostic Instruments, Sterling Heights, MI).

The intensity and location of tissue staining were assessed by a comparison with the positive and negative controls. Staining was considered positive (+++) only if the location and intensity of staining was consistent with that of the control melanoma tissue with 67–100% of cells staining positive. Tissue was considered negative if there was a definitive absence of staining. Slides were considered sporadic staining (+) if 1% to 33% of cells stained positive or intermediate staining (++) if 34% to 66% of cells stained positive. Tissue was assessed and graded by two independent observers blinded to the timing at which the specimens were obtained (initial biopsy, definitive surgery, recurrence). Specimens with discordant assessments were re-reviewed by the two observers and a consensus was reached.

Cell-Based ELISA

Twenty four short term cultures (OS71, OS191, OS194, OS223, OS229, OS231, OS233, OS238, OS242, OS248, OS268, OS269, OS290, OS291, OS297, OS300, OS301, OS303, OS307, OS308, OS314, OS319, OS322, OS323) described previously were used for ELISA.¹⁶ Ten thousand cells were plated in 96-well plates and incubated overnight (37°, 5% CO₂) in serum-free media (EMEM, 1% pen-strep, 1% non-essential amino acids). Cells were fixed with 10% buffered formalin and incubated at room temperature. Plates were washed and incubated with blocking solution (2% BSA in PBS). After blocking, plates were washed and incubated with primary antibody (100ng of 14G2a antibody [Santa Cruz Biotechnology, Santa Cruz, CA] while shaking slowly (50rpm on an I2400 New Brunswick incubator shaker). Following additional washes, secondary antibody was added (1:1000 dilution of HRP-conjugated anti-mouse IgG2a in PBS, Santa Cruz Biotechnology) and incubated. Plates were then washed and 100μL of TMB substrate was added to each well. After substrate turned blue, 100μL of stop solution was added (1M phosphoric acid). Plates were read at 450nm on a Benchmark Plus microplate reader (BioRad, Hercules, CA).

Standard Curve Preparation: Purified human GD2 protein (American Research Products, Belmont, MA) was plated in a 96-well plate at concentrations from 12.5 ng/well to 1600 ng/well. Plates were incubated overnight. The ELISA procedure was identical to that used for the osteosarcoma cells with the exception that no fixing step was performed.

Statistical Analysis

Results of the immunohistochemistry assays are reported as frequencies of negative staining (–), sporadic staining (+, 1–33% of cells stained positive), intermediate staining (++, 34–66% of cells stained positive), and positive staining (+++, 67–100% of cell stained positive) sorted by the timing at which the specimen was obtained (initial biopsy, definitive surgery, recurrence). Fischer exact test was used to determine the difference between intermediate and positive staining (++ and +++) versus negative and sporadic staining (– and +) in the recurrence samples versus the biopsy and definitive surgery samples. A p-value <0.05 was considered statistically significant.

RESULTS

Demographics

Forty-four human osteosarcoma tissue samples from 41 individual patients were available to evaluate GD2 expression. Eighteen percent of samples were obtained from the initial biopsy, 64% were obtained at the time of definitive surgery, and 18% were obtained upon relapse (Table I). One relapsed specimen was obtained from a metastatic bone lesion. All other specimens obtained at the time of relapse were resected lung metastases.

GD2 Distribution in Osteosarcoma Specimens

A tissue micro-array analysis with 44 samples was utilized. The samples were stained with 14G2a, a murine monoclonal anti-GD2 antibody, and the distribution and intensity of GD2 staining was qualitatively graded compared with positive and negative control samples (Figure I). There was 96% concordance between the independent observers.

The results of the tissue micro-array showed the presence of GD2 in 100% of the patient samples (Table I). No significant difference was seen in GD2 staining between samples obtained from the initial biopsy and samples obtained from the definitive surgery. However, the percent of cells staining positive for GD2 was significantly greater in tissue obtained at recurrence compared with tissue obtained at initial biopsy or during the definitive surgery. One hundred percent of samples obtained at the time of recurrence displayed intermediate or positive staining compared with 53% of samples obtained at the initial biopsy or definitive surgery ($p=0.016$).

GD2 Quantification

To evaluate GD2 expression quantitatively a cell based ELISA was performed on 24 osteosarcoma short term cultures (Figure II). Neuroblastoma and fibrosarcoma are malignancies known to express GD2. Thus, the neuroblastoma cell line, BE(2)-C, and the fibrosarcoma cell line, HT1080, were used as positive controls for this experiment. Nearly all of the osteosarcoma cell lines showed levels of GD2 expression comparable to the neuroblastoma and fibrosarcoma cell lines. Eighty-three percent and 46% of osteosarcoma (OS) cell lines expressed GD2 at higher levels than BE(2)-C and HT1080, respectively. Forty-six percent of OS cell lines expressed GD2 at a level $>2x$ that of BE(2)-C and 12.5% of OS cell lines showed $5x$ expression of BE(2)-C.

DISCUSSION

Over the past decade, the identification and targeting of proteins specific to cancer cells has become increasingly important to improving outcomes for patients with cancer. In recent years, targeted therapy such as imatinib, trastuzumab, and CH14.18 has improved survival for patients with acute lymphoblastic leukemia, breast cancer and neuroblastoma, respectively.^{4,5,17} Similar to this study, a prior study suggests GD2 is expressed in osteosarcoma.¹¹ In contrast to the prior study, this study utilizes an antibody which recognizes the same epitope as the therapeutic antibody, CH14.18 that will soon potentially be licensed by the Food and Drug Administration. Our findings that GD2 is highly expressed on the surface of osteosarcoma cells provide promising data supporting the development of clinical trials assessing the efficacy of anti-GD2 therapy in patients with osteosarcoma.

Our study showed that relapsed osteosarcoma specimens demonstrated higher expression of GD2 compared with samples obtained at initial biopsy and definitive surgery. This finding suggests that osteosarcoma cells with high expression of GD2 may be more resistant to

chemotherapy and provides additional support for targeting the antigen in clinical trials. In order to validate this finding, prospective studies, assessing changes in GD2 expression between primary and relapsed specimens from individual patients, will need to be conducted.

Numerous preclinical and clinical trials have assessed targeting of GD2 in patients with cancer focusing on two murine monoclonal antibodies, 3F8 and 14G2a and a human-mouse chimeric antibody, CH14.18.^{9,10,13,18–28} The majority of these studies have focused on patients with neuroblastoma and melanoma, due the high surface expression in these malignancies. A few patients with osteosarcoma have been included in early, phase I studies of GD2 antibodies. Variable responses were seen in these patients, and one patient with multiple bone metastases, treated with 14G2a + IL2 had a complete response. (Table II) The addition of IL2 and GM-CSF to anti-GD2 therapy demonstrated increased antibody dependent cell mediated cytotoxicity.^{29,30} A recent study by Yu, et al, showed Ch14.18, an anti-GD2 antibody, administered with GM-CSF and IL-2, improved survival for patients with high-risk neuroblastoma.²² Patients with no evidence of residual disease had the greatest benefit from the antibody therapy, and patients received the treatment after intensive induction chemotherapy, autologous stem cell transplant, surgery, and radiation therapy. These findings may have implications for the design of protocols incorporating CH14.18 in other malignancies which express GD2 on the cell surface.

Current anti-GD2 therapy induces cytotoxicity by stimulating the host immune system to clear tumor cells bound by antibody.^{29,30} It is difficult to assess the cytotoxicity of anti-GD2 therapy *in vitro* and osteosarcoma xenograft models are frequently in an immunosuppressed background. Thus, while it is feasible to show the antibody binds osteosarcoma cells, it is difficult to clearly assess tumor response and cytotoxicity. One potential approach will be to assess the effectiveness of anti-GD2 antibodies with cytokines in canine models of osteosarcoma, as the dogs have fully functional immune systems. These studies should address tumor response, time to progression, and overall survival in dogs with osteosarcoma treated with anti-GD2 antibody therapy. Additionally, it is unclear whether the GD2 antigen remains on the cell surface of osteosarcoma cells after treatment with anti-GD2 antibody similar to neuroblastoma.^{31,32} Canine studies should assess the persistence of surface GD2 antigen after antibody treatment, and could assess the utility of GD2 expression as a predictive biomarker.

The poor survival of patients with metastatic and recurrent osteosarcoma, despite decades of clinical trials, highlights the need for novel anti-cancer agents. Our finding that GD2 is highly expressed on osteosarcoma cells, paired with recent data showing the effectiveness of anti-GD2 therapy support the development of clinical trials in patients with metastatic and relapsed osteosarcoma.

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Figure I.
Representative immunohistochemical staining for GD2 showing: (A) positive control (melanoma tissue stained with 14.G2a, (B) negative control (melanoma tissue stained with IgG2a isotype control, (C) positive (+++) osteosarcoma sample, (D) sporadic staining (+) osteosarcoma sample.

FIGURE II.

GD2 expression in 24 osteosarcoma cell lines (c191-c323 and OS71). BE(2)-c (neuroblastoma cell line) and HT1080 (fibrosarcoma cell line) were used as controls. All 24 cell lines indicated the presence of GD2 cell-surface protein.

Immunohistochemistry analysis of GD2 protein expression in osteosarcoma samples. Samples were scored as follows:- (negative staining), + (sporadic staining), ++ (intermediate staining), +++ (positive staining). P value= 0.016 represents difference between intermediate and positive staining (++ and +++ versus negative and sporadic staining (- and +) in the recurrence samples versus the biopsy and definitive surgery samples.

Table 1

	n	-	+	++	+++
Biopsy	8	0 (0%)	4 (50%)	1 (12.5%)	3 (37.5%)
Definitive Surgery	28	0 (0%)	13 (46.4%)	8 (28.6%)	7 (25%)
Recurrence / Relapse	8	0 (0%)	0 (0%)	2 (25%)	6 (75%)
Total	44	0 (0%)	17 (38.6%)	11 (25%)	16 (36.4%)

Table II

Prominent clinical trials using anti-GD2 antibody.

Antibody	Trial Design	Disease (n)	Dose	MTD	DLT	Response	Reference
3F8 (murine monoclonal)	Phase I	NB(16) Melanoma(9)	5–100 mg/m ² , 2–4 days	50mg/m ²	HTN	NB: 2 CR Melanoma: 2 PR	Cheung 1987
	Phase II	NB(16)	10mg/m ² /dose×5 days	-	-	1 CR	Cheung 1988
	Phase II	NB (45)	10mg/m ² / day	-	Pain	12 CR (of 15) in BM in patients resistant to induction tx 5CR (of 10) in BM in patients resistant to retrieval tx	Kushner 2001
	Retrospective	NB(169)	10 or 20mg/m ² / day	-	-	PFS=62% and OS=81% for patients receiving 3F8 + GM-CSF + Cis-RA	Cheung 2012
14G2a (murine monoclonal)	Phase I	Melanoma(12)	10–120mg total divided days 1,3,5,8	80mg total	Neuro-toxicity	1 PR, 1 MR	Saleh 1992
	Phase I	NB(9)	100–400mg/m ² divided over 5–10 days	NR	NR	2 CR, 2 PR	Handgretinger 1992
	Phase I	NB(5) Melanoma(11) Osteo(2)	10,20,40 mg/m ² /day×5 days	15mg/m ²	Hypotension	NB: 2 PR Melanoma: 2 MR OS: 1 MR	Frost 1997
CH14.18 (human-mouse chimeric)	Phase I	Melanoma(13)	5–100mg, 1–2 days	50mg	Pain	None	Saleh 1992
	Phase I	NB(9)	20–50mg/m ² /dose×5 days	50mg/m ²	Pain, Urticaria	2 CR, 2 PR	Handgretinger 1995
	Phase I	NB(10) Osteo(1)	10–200mg/m ² over 1–4 days every 2–3 weeks	NR	Pain, Serum Sickness, HTN	NB: 1 PR, 4 MR	Yu 1998
	Retrospective	NB(166)	20mg/m ² /dose×5 days every 2 months	-	Pain, Capillary Leak, Pain	3yr OS improved vs Cis-RA alone vs, no tx, No	Simon 2004

Antibody	Trial Design	Disease (n)	Dose	MTD	DLT	Response difference in EFS	Reference
Hu14.18-IL2 (single agent fusion antibody)	Phase III (+IL2 + GM-CSF)	NB(226)	25mg/m2/day×4 days every 4 weeks	-	Pain, hypotension, capillary leak	2yr OS 86% vs 75% and EFS 66% vs 46% for experimental vs standard tx	Yu 2010
	Phase I	Melanoma(33)	0.8–7.5mg/ m2/ day	7.5mg/m2	Hypoxia, Hypotension	None	King 2004
	Phase I	NB(27) Melanoma(1)	2–14.4mg/m2/day	12mg/m2	Allergic Reaction, Myelo-suppression	None	Osenga 2006
	Phase II	NB(39)	12mg/m2/ day	-	Hypotension, Capillary Leak, Hypoxia	5 CR (of 23) in BM or MIBG only disease 0 of 12 in bulky disease	Shusterman 2008
	Phase II	Melanoma(14)	6mg/m2/ day	-	Hypotension, oliguria	1 PR	Albertini 2012

Abbreviations: BM, bone marrow; CR, complete response; DLT, dose-limiting toxicity; HTN, hypertension; MIBG, meta-iodobenzylguaniline scanning; MR, mixed response; MTD, maximum tolerated dose; NB, neuroblastoma; NR, not reported; OS, overall survival; EFS, event free survival; PFS, progression-free survival; PR, partial response; Osteo, osteosarcoma; cis-RA, cis-retinoic acid; IL2, interleukin-2; GM-CSF, granulocyte macrophage colony-stimulating factor.