Disialoganglioside \( G_{D2} \) Loss following Monoclonal Antibody Therapy Is Rare in Neuroblastoma

Kim Kramer, William L. Gerald, Brian H. Kushner, Steven M. Larson, Meera Hameed, and Nai-Kong V. Cheung

Departments of Pediatrics [K. K., B. H. K., N.-K. V. C.], Pathology [W. L. G.], and Nuclear Medicine [S. M. L.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021; and Department of Pathology, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103 [M. H.]

ABSTRACT

Ganglioside \( G_{D2} \) is abundant on human neuroblastoma (NB). Monoclonal antibody 3F8 targeted to \( G_{D2} \) may have imaging and therapeutic potential. Antigen-negative clones can escape immune-mediated attack, leading to clinical resistance or recurrence. Among 95 evaluable patients treated i.v. with 3F8 (94 stage 4 and 1 stage 3), 66 received nonradiolabeled 3F8, 11 received \(^{131}\)I-labeled 3F8 (8–28 mCi/kg) with autologous bone marrow rescue, and 18 received both forms of treatment. Prior to treatment, 91 patients tested positive for \( G_{D2} \) reactivity by bone marrow immunofluorescence (n = 68), tumor immunohistochemistry (n = 20), or diagnostic radioimmunoscintigraphy only (n = 3). Of 62 patients who had refractory or recurrent NB following 3F8 treatment, 61 (98%) tested positive for \( G_{D2} \) reactivity by bone marrow immunofluorescence (n = 51) or tumor immunohistochemistry (n = 10). The sole tumor that lost \( G_{D2} \) expression underwent phenotypic transformation into a pheochromocytoma-like tumor. The persistence of \( G_{D2} \) expression in refractory or recurrent NB suggests that complete antigen loss is an uncommon event and cannot account for treatment failure.

INTRODUCTION

Human NBs\(^1\) comprise a group of malignancies arising from neural crest precursors that differentiate along developmental pathways of the sympathetic nervous system (1), most commonly along gangliocytic and Schwannian lineages (2). Gangliocytic differentiation is a common occurrence in many treated and untreated NBs. Rarely, compound adrenal medullary tumors demonstrate the coexistence of both pheochromoblastic and neuroblastic lineages, wherein small foci of NB, ganglioneuroblastoma, and ganglioneuroma are admixed with adrenal pheochromocytoma (3). Such histopathological entities have resulted in the hypothesis that tumors derived from the neural crest are “pluripotent” and may be driven to differentiate along discrete lineages under appropriate conditions.

Gangliosides are sialic acid-containing glycosphingolipids that are mainly found in cell membranes. They have been recognized as important molecules related to the oncogenic transformation and cell differentiation of some human tumors (4). Ganglioside \( G_{D2} \) is highly expressed in NB but not in differentiated ganglioneuromas, neuroepitheliomas, or pheochromocytomas (5). \( G_{D2} \)-specific monoclonal antibodies 3F8 and 14.18 have been used in the detection and treatment of human tumors (6–8). As a target antigen, \( G_{D2} \) has many attractive properties, including its high tumor density, relative lack of modulation, and homogeneous tumor cell expression (8). However, it is possible that antigen-negative clones can escape immune attack by these antibodies leading to tumor progression or recurrence. We, therefore, assessed \( G_{D2} \) expression in the tumor samples of patients with NB before and after treatment with 3F8.

PATIENTS AND METHODS

All patients in this analysis had a histologically confirmed diagnosis of NB and were treated with anti-\( G_{D2} \)-monoclonal antibody 3F8 at Memorial Sloan-Kettering Cancer Center between 1987 and 1997. Patients were staged by the International Neuroblastoma Staging System (9). Disease status was generally determined prior to and at periodic intervals after antibody treatment by computed tomography or magnetic resonance imaging of previous or suspected sites of disease, \(^{99m}\)Tc-labeled bone scans, \(^{131}\)I-labeled-metaiodobenzylguanidine scans, bone marrow aspirations and biopsies, and urinary vanillylmandelic acid and homovanillic acid levels. Informed consent for treatment regimens was obtained in accordance with institutional review board guidelines.

Nonradiolabeled 3F8 was generally administered as a 90-min daily infusion (10 mg/m\(^2\)) for 5–10 days per course. Patients received one to four courses, based on serum antimouse antibody titers, such that the presence of serum antimouse antibody titers of >1000 units/ml precluded treatment. \(^{131}\)I-labeled 3F8 (8–28 mCi/kg) was administered over 5 days, followed by autologous bone marrow reinfusion. Eighteen patients received both forms of treatment.

\( G_{D2} \) Expression. \( G_{D2} \) expression was determined by immunostaining or by diagnostic imaging with \(^{131}\)I-labeled 3F8 prior to and periodically following 3F8 treatment, as described previously (10). Immunofluorescence of pooled bone marrow aspirations obtained from multiple iliac crest sites was used to

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\(^2\) To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, Department of Pediatrics, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-8401; Fax: (212) 744-2245; E-mail: Cheungn@mskcc.org.

\(^3\) The abbreviation used is: NB, neuroblastoma.
determine G\textsubscript{D2} expression in patients with histologically proven bone marrow disease. Fluorescein-conjugated affinity-purified goat F(ab')\textsubscript{2} antimouse immunoglobulin IgG and IgM were purchased from Biosource International (Camarillo, CA). A panel of anti-G\textsubscript{D2} monoclonal antibodies (3F8, 3G6, 3A7, 5E11, and 5F11) was used; stained marrow samples were examined using a fluorescent microscope and were scored as positive if homogeneous or bright surface staining was visualized, as described previously (11).

For patients with available frozen tumor samples, G\textsubscript{D2} expression was analyzed on 8-\mu m cryostat frozen tumor sections fixed in acetone. Endogenous peroxidases were blocked in 0.3% H\textsubscript{2}O\textsubscript{2} in PBS. Sections were incubated in 10% normal horse serum (Life Technologies, Inc., Gaithersburg, MD) and incubated with 3F8 (2 \mu g/ml) for 1 h. An IgG3 antibody, FLOPC 21 (Sigma Chemical Co., St. Louis, MO), was used as a negative control. Sections were incubated with a secondary antimouse biotinylated antibody (Vector Laboratories, Burlingame, CA), followed by incubation with the ABC complex (Vector Laboratories), and stained with the Vector VIP substrate (Vector Laboratories). A 10% hematoxylin counterstain was used. Staining was graded as positive or negative according to the presence or absence, respectively, of immunoreactivity.

RESULTS

Ninety-five of 115 patients treated with monoclonal antibody 3F8 were evaluable for G\textsubscript{D2}-positive disease by immunostaining of bone marrow or tumor samples or by immunoscintigraphy. All but two had stage 4 disease. Forty-one patients (43%) were evaluated at Memorial Sloan-Kettering Cancer Center at the time of initial diagnosis. Fifty-four patients (57%) had a histologically proven diagnosis of relapsed or refractory NB. Eighty-four patients received one to four courses of unlabeled 3F8 [1 cycle (n = 38), 1.5 cycles (n = 1), 2 cycles (n = 18), 3 cycles (n = 11), and 4 cycles (n = 16)]. Twenty-nine patients received \textsuperscript{131}I-labeled 3F8; this was the sole form of 3F8-targeted therapy for 11 patients.

Prior to treatment, 91 patients tested positive for G\textsubscript{D2} reactivity by bone marrow immunofluorescence (n = 68), tumor immunohistochemistry (n = 20), or diagnostic radioimmunoscinigraph only (n = 3; Fig. 1A). Four patients had no tumor specimens available for testing or no \textsuperscript{131}I-labeled 3F8 imaging prior to receiving 3F8. Thirty-three patients remained in remission. Of 62 patients who had refractory or recurrent NB following 3F8 treatment, 61 (98%) tested positive for G\textsubscript{D2} reactivity by bone marrow immunofluorescence (n = 51) or tumor immunohistochemistry (n = 10; Table 1). In general,
MYCN amplified. Despite multiple cycles of chemotherapy, she elusively elevated. A biopsy of the abdominal mass revealed a persistent disease in the retroperitoneum and spinal canal.

DISCUSSION

NB is the third most common malignant tumor of childhood and represents ~15% of all pediatric cancer-related deaths. The divergent clinical behavior of NB depends on the age and stage of the patient and a closely associated group of biological variables (12). Whereas prolonged survival (typically cure) is the rule with localized tumors, requiring little or no treatment, metastatic NB is often fatal, despite aggressive chemotherapy, surgery, and radiation therapy (13). NB disseminated to bone in children >1 year of age at diagnosis typifies the difficulty in curing metastatic cancers.

Various histological features are identified in neuroblastic tumors arising from the neuroectodermal cells of the adrenal medulla or the sympathetic ganglia (1). Differentiation toward gangliocytic and Schwannian lineages in vivo and in tissue culture mimics the clinical course of ganglioneuroblastomas or ganglioneuromas (14). In this series, we describe the persistent expression of GD2 in 61 of 62 patients with refractory or recurrent NB postimmunotherapy. The sole patient whose tumor lost GD2 expression demonstrated the unusual evolution of a histologically classic NB into a pheochromocytoma-like tumor.

As early as 1914, it was recognized that the neuroectoderm of the adrenal medullary tube gave rise to early neurocytes capable of differentiating toward the sympathetic system (ganglion cells and sheath cells) or chromaffin lineages (pheochromoblasts and pheochromocytes; Ref. 15). This model of neuroectodermal differentiation was supported by several observations in NB specimens with pheochromocytic and pheochromoblastomatous elements (15, 16). Specific chromaffin-related genes involved in normal adrenal medulla maturation (tyrosine hydroxylase and chromogranin A) are expressed in NB and support the capacity of early undifferentiated neuroblasts to undergo chromaffin cell differentiation (17). Sequential development of pheochromocytoma up to 15 years following treatment for NB has been described in case reports (18). In one instance, a mixed ganglioneuroma, NB, and malignant Schwannoma arose in the same location where a NB had been resected 18 years earlier (19). The only tumor that became GD2 negative in our series was initially a GD2-positive differentiating NB that evolved to a pheochromocytoma-like histology following immunotherapy. At the time of this patient’s death, all histological specimens showed evidence of neuroendocrine differentiation and were composed of paraganglionic/pheochromocytic elements without any remnants of either neuroblastic or ganglioneuroblastic lineages. The hepatic lesions prior to death that revealed fragments of small cells consistent with NB were GD2 negative support that the tumor was evolving phenotypically, with loss of GD2 expression being one of the first histopathological changes. The initial differentiation toward a ganglioneuroblastoma represents a common evolutionary pathway of NBs, but the reason for the altered paraganglionic/pheochromocytic maturation switch is unknown.

The effect of immunotherapy on phenotypic alterations of human tumors is not well studied. Antigen loss in melanomas has been described in association with T-cell responses in vivo (20). It is a relatively common observation among patients with B-cell lymphoma treated with anti-idiotypic monoclonal antibodies that mediate the selective killing of antigen-bearing clones (21). Monoclonal antibody 3F8 is specific for ganglioside GD2 and effectively kills tumor cells in the presence of human

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<th>Table 1</th>
<th>GD2 expression before and after treatment with 3F8</th>
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<td>Characteristics</td>
<td>n (%)</td>
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<tr>
<td>Patients treated with 3F8</td>
<td>115</td>
</tr>
<tr>
<td>Evaluable for GD2 pre/posttreatment</td>
<td>95</td>
</tr>
<tr>
<td>Stage 4 disease (bone marrow, bones)</td>
<td>93 (98)</td>
</tr>
<tr>
<td>Unlabeled i.v. 3F8 (1-4 cycles)</td>
<td>84 (88)</td>
</tr>
<tr>
<td>131I-3F8 i.v. (8-28 mCi/kg)</td>
<td>29 (31)</td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Patients in remission</td>
<td>33 (35)</td>
</tr>
<tr>
<td>Patients with recurrent/refractory NB</td>
<td>62 (65)</td>
</tr>
<tr>
<td>Samples remaining GD2 positive</td>
<td>61 (98)</td>
</tr>
<tr>
<td>Bone marrow immunofluorescence</td>
<td>51</td>
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<td>Tumor immunohistochemistry</td>
<td>10</td>
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although immunohistochemistry was not quantitative, tumor cells on frozen tissue sections demonstrated homogeneous GD2 expression.

One patient died of a GD2-negative tumor. She presented at age 34 months with stage 4 NB, involving a paraspinal mass, multiple bones, and bone marrow. Urinary catecholamines (vanillylmandelic acid and homovanillylmandelic acid) were markedly elevated. A biopsy of the abdominal mass revealed a stroma-poor, neuron-specific enolase-positive, S-100-positive, GD2-positive differentiating NB (Fig. 2A). The tumor was not MYCN amplified. Despite multiple cycles of chemotherapy, she had persistent disease in the retroperitoneum and spinal canal. An 131I-labeled 3F8 imaging scan showed widespread uptake (Fig. 1A). The patient received her first course of 31I-labeled 3F8 (12 mCi/kg), followed by one course of unlabeled 3F8. She underwent a laminectomy and a subtotal resection of the abdominal tumor revealing a GD2-positive ganglioneuroblastoma/differentiating NB without necrosis. The residual epidual disease was treated with radiotherapy, repeat surgery, and a second course of 131I-labeled 3F8 (12 mCi/kg). The resected specimen showed a GD2-positive ganglioneuroblastoma that was histologically similar to previous material (Fig. 2B). Nine months later, a routine bone scan revealed a left posterior parietal skull lesion, treated with surgical excision and radiotherapy. Progressive disease was found in the bone, bone marrow, and liver 6 months later, although bone marrow immunofluorescence was negative for GD2-positive cells. A biopsy of the hepatic lesions revealed fragments of malignant small cell tumor consistent with NB; the tumor cells, however, were negative for GD2. A 3F8 imaging scan was negative for GD2-positive disease (Fig. 1B). The patient died of disseminated disease shortly thereafter. At autopsy, histological examination of extensive tumors involving the abdomen, mesenteric nodes, liver, and lungs showed a diffuse neuroendocrine/paragangliocytic pheochromocytoma-like tumor, with finely granular cosinophilic cytoplasm, pleomorphic cells with vesicular nuclei, some distinct nucleoli, and some evidence of necrosis. No neuroblastic or ganglioneuroblastic features were present. Immunohistochemical analysis was strongly positive for chromogranin and neuron-specific enolase but negative for GD2 (Fig. 2C).
Fig. 2 Histological specimen of the sole tumor that lost GD2 expression. A, the abdominal mass before 3F8 treatment was a GD2-positive differentiating ganglioneuroblastoma with several neuroblastic clusters. Homogeneous GD2 reactivity on the frozen tissue was identified using the VIP (purple chromogen; Vector Laboratories). B, autopsy specimen demonstrated a GD2-negative paraganglionic differentiated tumor with a diffuse pattern of growth. No neuroblastic features were present. Corresponding hematoxylin (C) and eosin (D) sections are shown.

complement and by antibody-dependent cell-mediated cytotoxicity in the presence of human white cells (8). Disialoganglioside GD2 was expressed on all initial neuroblastic tumors prior to 3F8 therapy and was abundant in 98% of refractory/recurrent specimens postimmunotherapy. A given patient's immune status, human antimouse antibody status, and circulating GD2 may each contribute to differences in clinical and histological response. The sole patient whose tumor lost GD2 had extensive tumor/marrow samplings plus 3F8 imaging before and after 3F8 treatment. Antigen loss was documented by immunohistochemistry, marrow immunocytology, and immunoscintigraphy. This patient had no serum antimouse antibodies at the time of 3F8 imagings; thus, the absence of tumor uptake was not due to neutralizing antibodies. The absence of GD2 in the pheochromo-
mocytic cells is consistent with our observation that pheochromocytomas are generally G22-negative. Further 3F8 immunotherapy was also clinically ineffective in this patient. It is unlikely that the pheochromocytoma represented a second de novo or treatment-related malignancy, given the relatively short duration of her illness and a family history negative for endocrine tumors. We believe that the anti-G22 therapy eradicated the G22-bearing clones in this patient. The intensity of 3F8 treatment in this patient was exceptional; she was the sole patient in this series who received two courses of 131I-labeled 3F8 (total, 24 mCi/kg) separated by unlabeled 3F8 (total, 240 mg/m²). She represents a striking example of how antibody-specific therapy may totally eliminate antigen-positive clones.

More importantly, G22 appears to be tightly associated with the neuroblastic lineage, either posing as an obligatory marker or playing a critical cellular function. It is perplexing why only one patient developed an antigen-negative clone. We postulate that G22-negative NB is rare or nonexistent. The presence of pheochromocytoma lineage (G2-negative) NB is also uncommon. Because of persistent G22 expression in refractory or recurrent NB, we conclude that antigen loss is an uncommon event at our current 3F8 dose and schedule. A prospective study (i.e., N7 protocol) will investigate whether 131I-labeled 3F8, when combined with unlabeled 3F8 in a high-intensity fashion, can eliminate G22-expressing tumors.

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REFERENCES


Disialoganglioside G(D2) loss following monoclonal antibody therapy is rare in neuroblastoma.
