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Immune Therapies for Neuroblastoma

Fariba Navid[1,*], **Michael Armstrong**[2], and **Raymond C. Barfield**[2]

[1]Department of Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee; Department of Pediatrics, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee

[2]Division of Pediatric Hematology/Oncology, Department of Pediatrics, Duke University, Durham, North Carolina.

Abstract

Neuroblastoma, a solid tumor arising from developing cells of the sympathetic nervous system, is the most common extracranial tumor in children. The prognosis for high-risk neuroblastoma remains poor with conventional treatment, and new approaches are therefore being explored to treat this disease. One such alternative therapy that holds promise is immune therapy. We review here the recent advances in 4 types of immune therapy – cytokine, vaccine, antibody, and cellular therapy – to treat neuroblastoma. We present preclinical research and clinical trials on several promising candidates such as IL-12, dendritic cell vaccines, anti-GD2 antibodies, and allogeneic hematopoietic stem cell transplant. An optimal treatment plan for neuroblastoma will most likely involve multimodal approaches and combinations of immune therapies.

Keywords

Neuroblastoma; immune therapy; cytokine therapy; IL-12; vaccine therapy; antibody therapy; anti-GD2 antibodies; cellular therapy; allogeneic hematopoietic stem cell transplant

Introduction

Neuroblastoma is one of the most common solid tumors in children, accounting for 8–10% of all childhood cancers. Approximately 600 new cases are diagnosed in the United States per year.[1] Most patients with neuroblastoma are young (median age at diagnosis of 18 months) and commonly present with metastatic disease. More than 60% of patients have high-risk tumors that are likely to be incurable. Standard treatment uses multi-modal therapeutic approaches comprising chemotherapy, surgical debulking or excision of the primary tumor, radiotherapy, differentiating agents such as 13-*cis*-retinoic acid and autologous bone marrow transplantation.[2] Despite this aggressive approach to therapy, most patients with high-risk neuroblastoma have disease recurrence with metastatic foci resistant to multiple drugs, thereby necessitating the use of alternative approaches to treat this disease. One such alternative therapy is immune therapy, which comprises multiple complex elements. All these elements need to be studied in combination for patients to derive optimal benefits from immune therapy. The main immune therapies relevant to neuroblastoma are cytokine, vaccine, antibody, and cellular therapies. In this review, we present both clinical and research data that hold the promise of translational applicability.

*Correspondence to: Fariba Navid, MD, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, Tennessee 38105, Phone (901) 595-2573, Fax (901) 521-9005, E-mail: E-mail: fariba.navid@stjude.org.

Cytokine Therapy

Cytokines play a critical role in regulating the immune system, both by stimulating immune responses against antigen challenges and by guiding the host reaction over a range of responses from cytotoxic to immune tolerance. Neuroblastoma can be a potential target for the immune system. However, it has been well established that patients with neuroblastoma after receiving intensive therapy do not elicit optimal immune responses to antigen challenges. Therefore, exogenous cytokines may be used to stimulate patients' immune response against residual neuroblastoma.

Interleukin 12 (IL-12) has been tested for its antitumor activity in several studies and clinical trials. IL-12 is a potent activator of NK cells and T-lymphocytes, which play an essential role in antitumor immunity.[3] It also promotes the development of humoral immunity by stimulating CD4+ T-cells. Its role in immune recognition in neuroblastoma has also been well studied. Siapati *et al.*[4] examined the antitumor immune response to neuroblastoma cells transfected to express IL-12 and found that IL-12 provided a robust antitumor response as a single agent, and CD4+ and CD8+ T-cells but not NK cells are required for mediating the response. Redlinger *et al*.[5] further examined the role of IL-12 in neuroblastoma anti-tumor immunity and found that when neuroblastoma-bearing mice were inoculated with an IL-12–secreting neuroblastoma cell line or a dendritic cell (DC) vaccine, the IL-12 produced induced a potent antitumor response in the mice and CD8+ T-cells are required for this effect. However, unlike the study by Siapati *et al*., in their study NK cells were required for the early immune response. On the basis of these observations, Redlinger et al. proposed a mechanism of IL-12–induced apoptosis of neuroblastoma cells: IL-12 induces a strong NK cell response, leading to tumor cell lysis; NK cells then present the antigen to DCs, leading to CD8+ T-cell activation, which is further potentiated by IL-12. This mechanism bypasses the need for CD4+ T-cells, which is useful because neuroblastoma tumor cells tend not to express MHC class I on their surface, thereby limiting the activation of CD4+ T-cells.[6, 7] IL-12 suppresses the anti-apoptotic signaling molecule Akt and induces the pro-apoptotic mediators Fas/FasL, TNF receptor, and TRAIL.[8] These studies, taken together, support that IL-12 plays an active role in stimulating an antitumor response to neuroblastoma.

The prominent role of IL-12 in activating antitumor immune responses led to further investigations on the possible cytokine combinations that could augment the IL-12 response. In the study by Siapati *et al*.,[4] the combination of IL-12 and IL-2 was shown to produce the greatest antitumor effect – tumors were eradicated or inhibited in 91% of mice inoculated with the neuroblastoma cell line but in only 63% of mice vaccinated with IL-12 alone. As IL-2 alone had little effect on tumor growth, the authors concluded that IL-2 potentiates the IL-12 response by stimulating the expansion of the T-cells activated by IL-12. In another study, IL-12 was combined with IL-18 via a DC/neuroblastoma cell fusion vaccine.[9] The vaccine itself measurably increased interferon-gamma production, but the cotransfection of IL-12 and IL-18 led to a robust interferon-gamma response. The authors also showed that both NK and CD8+ T-cells are activated by the fusion vaccine. IL-18 potentiated the impact of IL-12 and increased the survival of mice injected with neuroblastoma tumor cells: no mice injected with both IL-18 and IL-12 had liver metastases whereas 50% of mice injected with IL-12 alone showed metastases. Mice inoculated with the combination vaccine had a significantly increased survival as well. These studies indicate that IL-12 is by itself is a potent stimulator of antitumor responses, but its combination with other cytokines can potentially improve its clinical impact. However, the optimal combination of cytokines remains to be determined.

IL-12 has been used to treat renal cell carcinoma and melanoma in adults. In phase I[10–13] and II[14] studies, up to 1.5 μg/kg of rhIL-12 at various dosing schedules was relatively well tolerated by patients, with fatigue, liver transaminitis, and decreased WBC count being the most common

side effects. Results from these clinical trials – although modest – support that IL-12 therapy can be useful to treat human malignancies. They indicate that cytokines such as IL-12 may be used to treat malignancies, but need to be combined with other immunotherapies such as antibody treatment or vaccines for optimal results.

Vaccine Therapy

Adoptive, or passive, immunotherapy involves the administration of antibodies, cytokines, or immune effector cells with the goal of directing an immune response to residual disease. Active immunity, in contrast, requires the host to develop a targeted immune response. Active immunity is advantageous as it helps the body develop a long-lasting immune response against tumor antigens, which may aid future tumor surveillance, as opposed to the more transient effects of passive immunity. In the first human trial of a tumor vaccine given to patients with B-cell lymphoma,[15] all 4 patients with follicular B-cell lymphoma who received a series of 3 or 4 injections of DCs exposed to autologous tumor antigens had positive cellular and humoral responses to the keyhole limpet hemocyanin protein. More importantly, all patients also had a cellular response to their tumor proteins, which was sustained for several months after the vaccinations, although the intensity declined over time. Clinically, 3 of 4 patients had a significant response to the vaccine, with 1 achieving complete response (CR). The promising results of this study have helped promote the potential for vaccine therapy in oncology. However, creating successful active immunotherapy for a particular tumor can be daunting. Current vaccine strategies have been successful to cure infectious diseases, but their use in oncology is hampered by the presence of tumor-derived immune evasion and immunosuppression. Despite these challenges, significant progress has been made in developing active immunotherapy options for neuroblastoma.

Developing an effective vaccine for neuroblastoma poses some unique challenges. Patients with advanced tumors such as neuroblastoma tend to be immunocompromised because of the intensive therapies given for their malignancy. Also, neuroblastoma cells, like many other tumors, have a low baseline expression of major histocompatibility complex (MHC) class I surface antigens and beta-2-microglobulin, which are critical for T-cell–mediated immune responses. Tumor cells also secrete immunosuppressive hormones such as Fas ligand, IL-10, TGFβ, VEGF, and gangliosides,[16–19] which lead to predominance of Th2 helper T-cells and promote immune tolerance. Such immunosuppression in tumor cells indicates the need to optimize the vaccination strategy to promote a robust immune response in order for this therapeutic modality to be successful.

Several cytokines have been shown to improve the immune response to tumor antigens. Redlinger *et al*.[20] demonstrated that transfection of DC cells to overexpress IL-12 improves the immune response to xenograft neuroblastoma tumors in mice. Peritumoral injection of IL-12–expressing DCs cells led to DC and T-cell colonization in the tumor microenvironment. Also, studies have shown that IL-12 production leads to the secretion of interferon-gamma, a potent T-cell activator,[21, 22] and that CD8+ T-cells and NK cells are important for the response. NK cells, unlike T-cells, have the advantage of producing a cytotoxic effect without costimuation. In the IL-12–secreting DC cell system, NK cells appeared to be responsible for the initial response and CD8+ T-cells for a more long-term immunity; interestingly, CD4+ T-cells were not required for a response in this model.

Given the challenges outlined previously, several groups sought to further optimize vaccine therapy in neuroblastoma. Different vaccine types have been used, all attempting to improve the immune response by modulating cytokine production. Huebener *et al*.[23] created a DNA vaccine to target tyrosine hydroxylase, an enzyme highly expressed in neuroblastoma. Peptides were designed using three-dimensional computer models of the peptide-MHC class I complex

to identify those with the highest affinity. The vaccine was delivered orally using attenuated *Salmonella typhimurium* as a carrier, which helps stimulate the immune response through the presence of lipopolysaccharide on the bacterial membranes and CpG motifs within the bacterial genome known to activate Toll-like receptors.[24] Administration of this vaccine, both prophylactically and therapeutically, decreased tumor growth and prevented metastatic spread of neuroblastoma to the liver. The immune response was specific to neuroblastoma, and there was no infiltration of T-cells into the adrenal medulla, another site of tyrosine hydroxlase expression. The authors attributed the absence of autoimmunity to the lack of inflammatory signals in the adrenal gland to trigger migration of reactive T-cells to the adrenal medulla. Fest *et al*. targeted GD2 in their neuroblastoma vaccine.[25] GD2 is a relatively tumor-specific ganglioside expressed on the surface of neuroblastoma cells, making it an ideal target for immune therapy. Unfortunately, since GD2 is a glycolipid, it does not evoke a robust immune response and functions as a T-cell–independent antigen, thus preventing a lasting response.[26] Thus, in order to induce a T-cell response, Fest et al. developed a mimotope DNA vaccine delivered via attenuated *S. typhimurium* that mimics the GD2 structure as a peptide. [25] The peptide created by the DNA vaccine was shown to be bound by the established murine anti-GD2 antibody 14G2a. Administration of the oral vaccine to mice caused a 1.4- to 1.6-fold increase in anti-GD2 serum response. Both humoral immunity and NK cell activation were induced, but no significant CD8+ response was elicited. Croce *et al*. engineered a genetically modified neuroblastoma cell line (Neuro2a) to express IL-21.[27] IL-21 stimulates the proliferation, cytoxic function, and interferon-gamma production of human CD8+ effector T-cells.[28, 29] When injected into immunocompetent mice, the IL-21–secreting neuroblastoma cells were unable to produce tumors whereas NOD-SCID mice produced tumors, indicating that IL-21–secreting cells are immunogenic. Furthermore, when injected into mice bearing disseminated neuroblastoma, the vaccine produced a significant increase in mean survival time, and 14% of the mice were disease free at day 100. In addition to promoting an immune response, the IL-21–secreting cells decreased tumor vascularity, an effect of IL-21 and resulting interferon-gamma production that has been demonstrated previously in breast cancer cells.[30] The CD8+ T-cells also played a role in rejection of tumor cells, indicating activation of cellular immunity. Together, these studies support the feasibility of creating a neuroblastoma vaccine, but also stress the importance of optimizing the response to the vaccine through concurrent stimulation of the immune system.

Promising preclinical data and encouraging results in adult trials led to phase I trials of tumor vaccines in children. Geiger *et al*. performed an initial trial of a DC vaccine in 15 pediatric patients with different types of solid tumors.[31] In this trial, DCs were harvested from patients by leukapheresis and then cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 to expand the population. The cells were then exposed to autologous tumor lysates before administering the DC vaccine. The patients tolerated the vaccinations well, the major side effect being only some local reactions. Although, this study was not designed to establish efficacy, 1 patient had a partial response (PR) and 5 others had stable disease. Of the 3 neuroblastoma patients, 2 exhibited stable disease after vaccination. There was also a positive delayed-type hypersensitivity response to autologous tumor lysates in 3 of 6 patients vaccinated. Given the favorable toxicity profile and encouraging results, the authors recommended that future vaccine studies be undertaken. A second neuroblastoma-specific phase I vaccine trial was performed[32] wherein DCs were harvested by leukapheresis, but instead of exposing cells to tumor cell lysates DCs were pulsed with autologous tumor RNA before administration. Once again, the vaccines were tolerated well, without significant side effects. Unfortunately, in this trial, no significant anti-tumor responses or clinical improvements were seen. In a different approach, Bowman et al. vaccinated children with autologous tumor transfected to express IL-2.[33] The vaccine was well-tolerated and 3 of 10 patients showed a response with the vaccine alone, with 3 more patients responding to the

vaccine followed by oral etoposide. Four of 9 evaluable patients showed tumor-specific immune responses.

These studies demonstrate that vaccines for children with solid tumors are feasible to create and are well tolerated. Unfortunately, the anti-tumor responses in the DC vaccine trials were not significant. However, in both trials, the DCs were not matured before administering to patients. Recent data have revealed that mature DCs stimulate a more robust immune response. Furthermore, vaccines in both trials were not directed at a particular target and the immune system was not stimulated with cytokines, as has been done in preclinical studies. The IL-2–secreting vaccine suggests that this method may augment the immune response to vaccines. The use of tumor vaccines has shown promise, and optimizing their development will result in more effective treatment of neuroblastoma.

Antibody Therapy

Anticancer monoclonal antibodies (mAbs) targeting specific antigens on the tumor surface are being increasingly used to treat solid and hematologic malignancies because of their long half-life, low toxicity, and high affinity and specificity. The anti-tumor effect of antibodies can be either dependent or independent of the immune system. Immune-mediated mechanisms include antibody-dependent cell-mediated cytotoxicity (ADCC); complement-dependent cytotoxicity; and the ability of mAbs to alter the cytokine milieu or enhance development of an active anti-tumor immune response. Nonimmune-mediated effects include blocking a survival signal for the cancer cell. Antibodies may also be used as targeting agents. When linked to drugs, radioisotopes, and toxins, they can kill tumors cells by delivering these agents at high concentrations directly to the tumor.[34, 35]

Of the potential immune therapies for neuroblastoma, the ones most established in humans are antibody therapies and antibody-based therapies (e.g., immunocytokine) directed against GD2. GD2 is uniformly expressed in neuroblastomas.[36] Its function is not completely understood, but it is thought to play an important role in the attachment of tumor cells to extracellular matrix proteins.[37] GD2 expression in normal fetal and adult tissues is primarily restricted to the central nervous system (CNS), peripheral nerves, and skin melanocytes, although protein expression has been seen in the stromal component of some normal tissues and white pulp of the spleen.[38–40] Because of the relatively tumor-selective expression combined with its presence on the cell surface, GD2 is an attractive target for tumor-specific antibody therapy. Several anti-GD2 antibodies have been developed for clinical use over the past 2 decades. These antibodies are reviewed in the section below.

Murine Anti-GD2 Antibodies

3F8—3F8 is a murine IgG3 monoclonal anti-GD2 antibody developed by Cheung and colleagues primarily to target neuroblastoma cells. This antibody has undergone extensive preclinical and clinical testing. In vitro studies suggest that tumor cell killing by 3F8 is mediated by human complement[41] and other human immune effector cells, including lymphocytes[42], neutrophils[43], and monocytes.[44]

In the phase I and II testing of 3F8 modest tumor responses were observed in patients with neuroblastoma.[45, 46] Toxicities observed included hypertension (dose-limiting), severe pain, fever, and urticaria. All patients tested developed human anti-mouse antibodies (HAMA) to 3F8. The limited antitumor activity seen in these initial clinical studies prompted further evaluation of 3F8 in a setting of minimal residual disease.[47] Thirty-four patients (23 in CR, 8 in very good PR, and 1 in PR by conventional methods as per the International Neuroblastoma Staging System[48]) were treated with 3F8 for up to 4 courses based on disease status and HAMA titers. Of the 13 patients who remained disease-free 40–130 months after the first 3F8 treatment,

11 had disease confirmed by either conventional methods ($n = 3$) or non-conventional methods ($n = 8$) at the start of antibody therapy. Non-conventional methods included immunoscintigraphy using 3F8 radiolabeled with iodine-131 (^{131}I-3F8), bone marrow immunocytology, and molecular detection of the residual neuroblastoma marker GAGE by reverse transcriptase polymerase chain reaction (RT-PCR) in the bone marrow. In the entire cohort of 34 patients, evidence of response by immunocytology was noted in 6 of 9, by GAGE RT-PCR in 7 of 12, and by ^{131}I-3F8 immunoscintigraphy in 6 of 6 patients. These findings suggest that antibody therapy is beneficial in the setting of minimal disease burden.

Combining 3F8 and GM-CSF is a strategy that might enhance phagocyte-mediated antibody-dependent cellular cytotoxicity. When this combination was evaluated in 45 patients with high-risk neuroblastoma, the side effects were found to be manageable and the treatment appeared to benefit patients with bone marrow disease but not those with progressive disease and soft-tissue masses.[49] The investigators of this trial studied polymorphic alleles in the *FCGR2A* gene that encodes the Fcγ receptors (mediates ADCC and complement-dependent cytotoxicity) in patients who received 3F8 plus GM-CSF (n = 136).[50] Patients with the FCGR2A-R/R genotype had a better outcome than those with FCGR2-R/H, FCGR2-H/H, or FCGR3A genotype. A similar analysis in a smaller cohort of patients who received 3F8 alone suggested that the effects of *FCGR2A* on outcome were probably due to the addition of GM-CSF. A better understanding of the molecular basis for the activity of monoclonal antibodies in the presence or absence of cytokines will not only facilitate patient selection for these therapies but also shed light on ways to improve cytotoxicity.

^{131}I-3F8 has been used for imaging of neuroblastoma. 3F8 does not cross the blood-brain barrier when administered systemically. Applications of this antibody for CNS disease are limited to direct administration of the antibody into the cerebrospinal fluid. In a study evaluating the safety of intrathecal administration of ^{131}I-3F8, 15 patients with CNS and leptomeningeal malignancies were treated.[51] The dose-limiting toxicity was increased intracranial pressure and chemical meningitis. Other transient side effects included headache, fever, and vomiting. Three of 13 evaluable patients had an objective radiographic or cytologic response, or both.

14G2a—14G2a is a murine monoclonal anti-GD2 antibody unrelated to 3F8. This antibody is an IgG2a-class switch variant of 14.18, an anti-GD2 antibody of an IgG3 isotype. In phase I trials of 14G2a mAb[52, 53] evaluating two different dosing schedules, toxicities observed included pain, diarrhea, hyponatremia, paresthesias, hypotension, and allergic reactions (fever, rash, dyspnea, and hypoxia). Similar to 3F8, limited tumor responses were observed and HAMA titers were detected in the majority of patients.

On the basis of preclinical studies demonstrating an increase in ADCC of tumor cells in response to antibody treatment when IL-2 or GM-CSF is administered to enhance effector cell function,[54] a phase I study of 14G2a with either IL-2 alone or with both IL-2 and GM-CSF was undertaken by the Children's Oncology Group (COG).[55] Accrual to the regimen containing both IL-2 and GM-CSF was discontinued when adult patients receiving this combination of cytokines developed neurologic toxicity coinciding with hyperleukocytosis. In combination with IL-2, dose-limiting toxicities included grade 4 thrombocytopenia, hyperbilirubinemia, diarrhea, neutropenia, bronchospasm, tachycardia, hypotension, angioedema, and generalized pain. One patient with neuroblastoma had a PR, and 1 patient with osteosarcoma had a CR. Nine of 21 patients developed a HAMA response.

Although administration of 14G2a has proved feasible and antitumor effects are encouraging, one drawback of 14G2a and other murine mAbs for human use is the development of HAMA titers, which limit further antibody therapy and potentially contribute to the observed

hypersensitivity reactions. These concerns and new advances in genetic engineering have prompted the development of a human–mouse chimeric anti-GD2 antibody.

Human–Mouse Chimeric Anti-GD2 Antibodies

Ch14.18—Ch14.18 is an antibody in which the human Fc constant regions of an IgG1 immunoglobulin are fused with the Fab portion of the murine 14G2a antibody.[56] It retains the anti-GD2 specificity and the ability to target GD2-positive tumors and is 50–100 times more efficient at mediating tumor ADCC in vitro than is murine 14G2a.[57]

Phase I testing of ch14.18 was first performed in 13 adult patients with metastatic melanoma.[58] The dose chosen as the maximum daily dose was 50 mg, because abdominal or pelvic pain during antibody infusion precluded the use of higher doses. No other neurologic side effects or other severe toxicity occurred. Eight of the 13 patients developed antibodies to ch14.18, but the observed titers were only approximately 10% of those detected in the trials of murine 14G2a. Although no antitumor responses occurred, antibody was detected on tumor cells through fluorescence-activated cell sorting analysis in some of the patients treated with 45 mg or more of ch14.18.

In a pediatric phase I trial, 9 children (ages 2–10 years) with neuroblastoma received up to 50 mg/m^2 of ch14.18 for 5 days.[59] Pain was the most common side effect during treatment and was most pronounced at 50 mg/m^2, which was considered the MTD. Other side effects included fever, urticaria, pruritus, and rash. One patient developed transient pupillotonia at the highest dose. Optic nerve atrophy was observed in 2 patients, both of whom had received prior radiotherapy, which was implicated in the adverse event. This toxic effect gradually resolved in the 6 months after therapy with ch14.18. HAMA was not detected in any of the participants. Two of the participants had a CR, 2 a PR, and 1 a minor response.

An alternative dosing schedule of ch14.18 was tested in the phase I setting in 11 patients (10 with neuroblastoma and 1 with osteosarcoma).[60] The most common toxicities were pain, tachycardia, hypertension, fever and urticaria. An MTD was not established in this study, and the dosage was not further escalated because the supply of antibody had been exhausted. However, at the highest dose levels tested (100 and 200 mg/m^2; total doses over 2–4 days), pain was graded as severe in 5 of 7 patients. Serum sickness was observed in 2 of 4 patients who received 200 mg/m^2 ch14.18. Anti-ch14.18 immune response was detectable in 7 of 10 patients. Of 10 patients evaluable for response, 1 had a PR, 3 mixed responses, and 1 stable disease.

In the Cooperative German Neuroblastoma Trials NB90 and NB97 for patients with newly diagnosed high-risk neuroblastoma, ch14.18 was administered in the maintenance phase of treatment.[61] Of the 334 evaluable patients in these trials, 166 received mAb ch14.18, 99 received a 12-month course of low-dose maintenance chemotherapy, and 65 had no further treatment after initial therapy. The mAb was administered over a period of 1 year at a schedule of 20 mg/m^2/day for 5 days every 2 months (6 courses total). There was no statistically significant difference in event-free survival among patients treated with mAb ch14.18, maintenance chemotherapy, or no therapy after initial treatment. However, overall survival was better in the ch14.18 group (3-year overall survival, 68.5 ± 3.9%) than in groups receiving maintenance chemotherapy (3 year overall survival, 56.6 ± 5%) or no further therapy (3-year overall survival, 46.8 ± 6.2%).

As with other antibodies, ch14.18 has been evaluated in combination with GM-CSF and IL-2 in many clinical trials. In a phase I study of 24 adult patients with melanoma[62], the MTD of ch14.18 was 7.5 mg/m^2/day daily for 5 consecutive days in combination with IL-2 continuous infusion 1.5 million units/m^2/day for 4 days/week for 3 weeks. Dose-limiting toxicities were

severe allergic reaction in one patient and weakness, pericardial effusion, and decreased performance status in another. In a COG study, phase I evaluation of ch14.18 administered in combination with GM-CSF was performed in 19 neuroblastoma patients after autologous stem cell transplantation.[63] The MTD of ch14.18 in this combination was 40 mg/m^2/day daily for 4 days with GM-CSF (250 μg/m^2/day) started 3 days before and continued 3 days after. Ten of the 19 patients experienced disease progression, with a median follow-up of 40 months (range, 25–50 months).

Phase I studies revealed manageable toxicities of ch14.18 combined with cytokines IL-2 and GM-CSF, prompting the ongoing clinical trial in the COG for patients with newly diagnosed high-risk neuroblastoma. Patients in this study have been randomized to receive standard maintenance therapy with cis-retinoic acid versus *cis*-retinoic acid plus ch14.18 in combination with IL-2 alternating with GM-CSF.

Immunocytokines

Immunocytokines have been developed with the goals of enhancing the efficacy of antibody therapy and minimizing the systemic toxicities associated with the addition of cytokine therapy. Immunocytokines are fusion antibodies in which the Fc end of the monoclonal antibody is linked to a cytokine. The antibody binds to the target of interest on the tumor cell and delivers high concentrations of the cytokine directly to the tumor microenvironment to attract the immune effector cells required to kill tumor cells. Initially, ch14.18 was linked to IL-2 (ch14.18–IL-2) and this fusion protein retained the binding specificity of ch14.18 and the IL-2 component stimulated proliferation of IL-2–responsive cells.[64, 65] In GD2-positive murine neuroblastoma models, ch14.18–IL-2 eradicated metastatic neuroblastoma more efficiently than antibody administered with exogenously delivered IL-2.[66]

A humanized hu14.18 (98% human derived) was developed to reduce immunogenicity. This protein was genetically linked to IL-2 (hu14.18–IL-2, EMD 273063). The first phase I study of this fusion protein was performed in 33 adults with melanoma.[67] Common toxicities included fever and chills (100%), pruritus (61%), hyperglycemia (55%), hypophosphatemia (39%), and transient neuropathic pain (39%). Dose-limiting toxicities included hypoxia and hypotension, which were believed to be IL-2 related. The MTD was 7.5 mg/m^2/day. There were no objective responses, but there was prolonged disease stabilization in 4 patients with high-risk disease.

Phase I testing of hu14.18–IL-2 in children was conducted through the COG.[68] Twenty-seven patients with neuroblastoma and 1 patient with melanoma were treated. The MTD was 12 mg/m^2/dose. The investigators suggested that the higher MTD in pediatric patients may be related to more intensive prior immunosuppressive therapy received. Toxicities were similar to those reported in the phase I study on adults. No objective responses were observed.

Phase II testing of hu14.18–IL-2 has been performed in patients with neuroblastoma.[69] Thirty-nine patients received 12 mg/m^2/day hu14.18–IL-2 for 3 consecutive days every 28 days and were evaluated on the basis of disease measureable by standard radiologic criteria (n = 12) or by meta-iodo benzylguanine scanning or bone marrow histology (n = 23). No responses were observed by standard radiologic criteria; however, in the other group, 5 patients had a CR, suggesting a benefit of this therapy in the setting of minimal residual disease. Most toxicities were expected and reversible (pain, rash, allergic reaction, fever, and hepatic transaminitis). Of note, 2 patients required dopamine for hypotension and 1 required ventilatory support for capillary leak syndrome and hypoxia.

Anti-GD2 antibodies have shown promising anti-tumor activity in children with neuroblastoma. However, despite advances in antibody technologies to humanize these

antibodies and adding cytokines to boost the response of immune effector cells, the therapeutic potential of these antibodies remains to be optimized. Strategies to improve the efficacy of these antibodies have included GD2-targeted liposomes[70] and anti-GD2 antibodies in combination with novel chemokines[71] and cytokines,[72] as well as further modifications in the structure of the antibody.

A major breakthrough in antibody engineering has been the derivation of single-chain molecules called scFv fragments and dimeric single-chain antibodies called minibodies, or small immunoproteins (SIPs). ScFv fragments consist of the variable heavy chain and the variable light chain joined by a flexible linker. These molecules maintain their antigen-specificity, yet, because of their size, can extravasate more efficiently than an IgG molecule and diffuse more readily within tumors but are also rapidly eliminated through the kidneys.[73] These molecules can be conjugated to toxins, radioisotopes, or effector molecules. Pretargeting of neuroblastoma xenografts in mice with an anti-GD2 scFv fragment ligated to streptavidin (5F11-scFv-SA) has been shown to improve the tumor-to-nontumor ratio of biotinylated radionucleotides and polypeptides.[74] SIPs that are constructed by connecting an scFv to the dimerizing domain of human immunoglobulin γ1 chain penetrate tissues better than IgG molecules do, yet have a slower clearance than scFv fragments. Two anti-GD2 SIPs have been generated – one is a fully murine molecule containing the CH3 domain of mouse IgG1 and the other is a hybrid mouse–human molecule containing the CH4 domain of human IgE.[75] If the kinetics of these molecules prove to be favorable, these molecules alone or conjugated to other molecules may improve the therapeutic index of GD2-targeted therapies.

Alterations in antibody structure to reduce undesirable immune effects and enhance desirable antitumor effects have also been developed. At the St. Jude Children's Research Hospital, we are currently evaluating in children and young adults with refractory/recurrent neuroblastoma or melanoma the safety of a modified version of the hu14.18 antibody (hu14.18K322A), which has a single point mutation in the CH2 domain of the antibody. This region of the antibody has been shown to be critical for antibody-dependent complement activation.[76] Minimizing complement activation may ameliorate some of the side effects observed in patients receiving 14.18 antibodies, such as pain, fever, rash, and capillary leak syndrome, which are probably due to increased production of inflammatory peptides such as C3a and C5a. These toxicities limit the dose of antibody that can be given. One potential drawback of decreasing complement activation is that it may reduce the antitumor response of the antibody. However, Imai and colleagues[77] have shown in GD2-expressing–tumor-bearing wild-type, complement-deficient, complement-receptor–deficient, and Fcγ receptor I/III-deficient mice treated with anti-GD2 antibody 14G2a that the absence of complement does not appear to affect the anti-tumor effect of anti-GD2 antibody except at low concentrations of the antibody. By contrast, ADCC (absent in Fcγ receptor I/III-deficient mice) is required to eradicate tumors in the presence of antibody. In addition to the new design elements (i.e., point mutation K322A) incorporated into the hu14.18 molecule, the hu14.18K322A is expressed in a YB2/0 cell line. Because of the lack of fucosylation activity in these cells, anti-human antibodies produced by YB2/0 cell lines have higher levels of ADCC activity than antibodies produced by Chinese hamster ovary cell lines.[78, 79] In vitro, hu14.18K322A demonstrates less complement activation than ch14.18 and has comparable or better dose-dependent ADCC activity than ch14.18 and hu14.18 antibodies (unpublished data), suggesting that this antibody has the potential to be less toxic (thereby allowing administration of higher doses) and more effective.

The development of anti-GD2 antibodies has been challenging; however, the observed antitumor responses have driven further research to improve this therapy. As with other antibodies developed for therapeutic use, the promise of antibody therapy lies in treating minimal residual disease. These studies require large numbers of patients over an extended period of time to assess the benefit of treatment in this setting.

Cellular Therapy

Allogeneic transplantation was attempted in children in the 1980s for neuroblastoma, but the outcome was poor in part because of high transplant-associated mortality and graft versus host disease (GvHD). Reports of allogeneic transplantation for other pediatric tumors have been anecdotal. However, the renewal of interest in allogeneic transplantation for solid tumors in adult patients has led to several pilot studies which have indicated the presence of the graft versus tumor (GvT) effect. This has generated interest in the cellular immune therapies for neuroblastoma, especially because supportive care for patients undergoing allogeneic hematopoietic stem cell transplant (HSCT) has improved considerably. In patients for whom allogeneic HSCT has been effective in treating solid tumors, the GvT effect has usually accompanied GvHD.

The existence of a graft-versus-leukemia (GvL) effect has been amply supported by both experimental and clinical data, but less is known about the GvT effect in solid tumors. In allogeneic transplantation, specific host cells are recognized as foreign by donor-derived alloreactive T-cells, which results in GvHD. Antigens derived from broken-down proteins are presented on the surface of antigen-presenting cells by class I or II HLA molecules. In the context of an MHC identical donor and recipient, GvHD results when minor histocompatibility antigens are bound and presented in the clefts of MHC molecules, thereby leading to activation of donor T-cells. This basis for GvHD is also one way of achieving a GvT effect.

Cytotoxic T-lymphocytes can target several antigens found on malignant cells. These antigens include lineage-restricted antigens found on tumors of similar origin and on related normal cells (e.g., GD2 expressed on neuroblastoma), antigens found on tumors of different origins but not on normal tissue, and tumor-specific antigens produced by mutant genes within the tumor. The observed association of GvHD with GvT effects probably occurs when tumor cells and normal host cells share the target antigen. However, because tumors also express antigens that are unique to the tumor cells, or have a much higher expression of the target antigen than the normal tissue does, it is possible to have GvT effects without GvHD.

In light of the new potential of combined immune therapies, the current results of using alloimmune cellular therapies for neuroblastoma are not sufficient to draw firm conclusions. Two studies in the mid-1990s suggested that allogeneic HSCT is not superior to autologous HSCT, which is the current standard of care for high-risk neuroblastoma. The first study was a case control study of allo-HSCT for children with advanced or poorly responding neuroblastoma, using data from the European Bone Marrow Transplant Solid Tumor Registry.[80] Seventeen patients receiving allogeneic and 34 receiving autologous HSCT were matched for several prognostic factors, including age, sex, prior treatment duration, pre-graft response status, and bone and bone marrow involvement before HSCT. The progression-free survival (PFS) for the allogeneic and autogeneic HSCT groups was not significantly different − 35% and 41% at 2 years, respectively. Only half of the patients receiving allogeneic HSCT developed GvHD: 7 of 9 grade I-II and only 2 of 9 grade IV. The authors speculated that the absence of risk factors for GvHD in young children could be a major obstacle in achieving an anti-tumor effect with allogeneic HSCT in neuroblastoma. A second study reported in the same year compared the toxicity, relapse rate, and PFS of high-risk neuroblastoma patients receiving identical induction therapy and myeloablative chemotherapy plus total-body irradiation followed by allogeneic or autologous purged HSCT.[81] Patients with human leukocyte antigen (HLA)–compatible siblings received allogeneic bone marrow ($n = 20$). The remaining patients ($n = 36$) received autologous bone marrow that had undergone multimodality purging and had no remaining detectable tumor cells by immunocytology. Four of 20 patients receiving allogeneic HSCT had a treatment-related death compared with 3 of 36 patients receiving autologous HSCT. The relapse rate among patients receiving allogeneic HSCT was 69%,

compared with 46% for those receiving autologous HSCT ($P = 0.14$). The estimated PFS rates 4 years after HSCT were 25% for patients receiving allogeneic HSCT and 49% for those receiving autologous HSCT ($P = 0.051$). The overall outcome for patients with neuroblastoma given this same induction therapy followed by autologous purged marrow was similar to that for patients given allogeneic marrow.

The new concepts of allogeneic HSCT for neuroblastoma and other solid tumors do not rely on increasing the chemotherapy intensity and reducing tumor load but rather on the GvT effect.[82] A recent case report suggests that that novel approaches to allogeneic cellular therapies might be useful in treating chemo-resistant neuroblastoma. Inuoe et al. reported the case of a 5-year-old boy who received CD34-positive HLA haplo-identical HSCT from his father as treatment for refractory advanced neuroblastoma.[83] He had residual disease in the para-aortic lymph nodes and multiple bones after the transplant. He developed grade I acute GvHD but had no symptoms of chronic GvHD or any other complications. All his residual disease disappeared completely and he remained disease-free 3 years later. This case suggests the possibility of a GvT effect against neuroblastoma by HLA-mismatched allogeneic HSCT. A subsequent feasibility study using haplo-identical HSCT with T- and B-cell depletion was conducted by Lang et al.[84] Six patients with relapsed metastatic neuroblastomas ($n = 4$), rhabdomyosarcoma ($n = 1$) or Ewing's sarcoma ($n = 1$) after previous autologous transplantation received CD3/CD19-depleted grafts from mismatched family donors. There was no transplant-related mortality. At the time of reporting the median survival time was 6 months (2–11), though this data is difficult to interpret with low numbers and patients with advanced disease. Interestingly, analysis of post-transplant NK cell function revealed stable cytotoxic activity against K562 targets, whereas activity against neuroblastoma targets was low. However, stimulation with cytokines and use of appropriate antibodies clearly enhanced specific lysis in vitro, suggesting that if cellular therapy proves useful in the future, it may well be used as part of multimodal immune therapies.

More recently, Yoshida et al. reported a 7-year-old male with neuroblastoma who received an ex vivo–expanded donor CD4(+) T lymphocyte infusion after recurrence in the bone marrow following allogeneic HSCT from his HLA-identical mother.[85] The disease transiently responded to CD4(+) donor lymphocyte infusion, with reduction of tumor cells and a decrease of serum neuron-specific enolase. The response was associated with development of continued high fever and an increase of cytotoxic T lymphocytes in the peripheral blood. This case again suggests the possibility of a GvT effect against neuroblastoma, although how this effect can be used optimally is still unclear.

Conclusions

Current conventional therapies have proven inadequate in treating advanced disease in neuroblastoma. Pre-clinical research has broadened our understanding of the immune response to these tumors and guided the optimization of possible directed immunotherapies in the form of antibodies, vaccines, cytokines and cellular therapies. However, based on the modest antitumor responses of the individual therapies discussed in this review, the future success of immunotherapy for neuroblastoma almost certainly involves combining these treatment modalities and applying them in the setting of minimal residual disease.

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List of Abbreviations and Acronyms

ADCC, Antibody-dependent cell-mediated cytotoxicity
COG, Children's Oncology Group
CNS, Central nervous system
CR, Complete response
DC, Dendritic cells
GD2, Disialoganglioside
GM-CSF, Granulocyte-macrophage colony stimulating factor
GvHD, Graft-versus-host disease
GvL, Graft-versus-leukemia
GvT, Graft-versus-tumor
HAMA, Human anti-mouse antibody
HLA, Human leukocyte antigen
HSCT, Hematopoietic stem cell transplant
IL, Interleukin
mAb, Monoclonal antibody
MHC, Major histocompatibility complex
MTD, Maximum tolerated dose
NK, Natural killer
PFS, Progression-free survival
PR, Partial response
SIPs, Small immunoproteins
TGFβ, Transforming growth factor beta
TNF, Tumor necrosis factor
VEGF, Vascular endothelial growth factor

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