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Cellular Immunotherapy for Neuroblastoma: A Review of Current Vaccine and Adoptive T Cell Therapeutics

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Abstract

Immunotherapy is an attractive option for patients with high risk neuroblastoma due to their poor long-term survival rates after conventional treatment. Neuroblastoma cells are derived from the embryonic neural crest and therefore express tumor antigens not widely seen in normal cells, making them potential targets for immunologic attack. There is already considerable experience with monoclonal antibodies that target these tumor associated antigens, and in this review we focus on more exploratory approaches, using tumor vaccines and adoptive transfer of tumor-directed T cells.

Keywords

Neuroblastoma; immunotherapy; adoptive T cell transfer; chimeric antigen receptors; vaccine trials

INTRODUCTION

Over the past 20 years, efforts to improve the outcome for patients with high-risk neuroblastoma have focused on dose-intensifying chemotherapy, to levels that induce myeloablation and require autologous stem cell rescue. Biologic therapies with 13-cis-retinoic acid have also been added [1–5]. Unfortunately, despite improved remission rates, long-term survival in this population remains less than 40% [5, 6]. The limitations of current therapies have increased interest in experimental treatment options such as tumor-directed immunotherapies. Three types of immunotherapy have been used for patients with neuroblastoma: monoclonal antibodies, vaccination, and adoptive cellular therapy (Fig. 1).

MONOCLONAL ANTIBODY THERAPY

Monoclonal antibodies (MAbs) recognize tumor specific antigens and recruit effector mechanisms that kill tumor cells, usually by antibody dependent cell mediated cytotoxicity or complement mediated lysis. These effector mechanisms can be supplemented by coupling MAbs to toxins or radionuclides [7], or to "linker" molecules to bind them to effector T

cells [8]. Antigens recognized by monoclonal antibodies must be present on the cell surface and may be almost any type of molecule including proteins, carbohydrates, lipids, or a combination thereof. Antigens currently used as targets for monoclonal antibodies are not expressed exclusively on tumor cells [7, 9]; "on-target but off-target-organ" adverse-effects of MAb may therefore be significant, depending on the degree and distribution of antigen expression on normal cells. In addition, many MAb contain peptide sequences or entire protein chains remaining from their rodent origin, and may induce an immune response leading to serious hypersensitivity reactions [10].

Monoclonal antibodies targeting neuroblastoma tumor associated antigens (TAA) have been used since the mid 1980's [11] and were extensively reviewed by Johnson *et al.* in 2007 [7]. Tumor associated antigens targeted by MAb include the gangliosides GD2, (see Adoptive T Cell Therapy below) GD3, and GM3 and the glycoproteins CD56, L1-CAM, and GP95 [7, 12]. Clinical responses, including complete remissions, have been seen when monoclonal antibodies have been used alone [13–15]. In addition to single center studies, the Children's Oncology Group has sponsored Phase I, II, and III studies using MAb to treat relapsed/refractory disease, as well as incorporating these agents after front-line myeloablative chemotherapy and stem cell rescue. Hence, as in adult malignancies, it appears likely that MAb will come to have a defined and significant role in the standard treatment of high-risk neuroblastoma.

VACCINE THERAPY

Developing an effective vaccine for neuroblastoma is a considerable challenge. While vaccines have been extremely successful at preventing infectious diseases, they are much less beneficial once illness is established. With only a few exceptions, cancer vaccines will be used after patients have developed their malignancies, and not for cancer prevention. For those with established disease, the generation of sustained and effective anti-tumor immune responses to TAA may be difficult [16]. In patients with neuroblastoma, development of a safe and effective vaccine for generalized use may be further hampered by the heterogeneity of tumor pathobiology, since some malignancies undergo spontaneous regression, while others are highly metastatic and minimally responsive to most intensive therapies. Moreover, downregulation of major histocompatibility complex (MHC) and co-stimulatory molecules by neuroblastoma cells may limit the effectiveness of any tumor-specific T cell immune response induced by the vaccine [17–20].

Despite obstacles to developing a successful vaccine therapy for neuroblastoma, a number of studies have been reported, and tumor responses, including complete remissions, have been observed. In part because of the heterogeneity of the disease, neuroblastoma tumor vaccines have been composed of cellular extracts or whole cell products. These complex products have the advantage of allowing multiple tumor antigens to be presented and investigators have used autologous or allogeneic neuroblastoma tumor cells, dendritic cells (DCs) loaded with peptides, mRNA, or tumor cell lysates (Table 1) [21–24]. Whole cell vaccines are amenable to genetic-modification as a way to enhance induction of an anti-tumor immune response [16, 18], but unlike peptide, protein or other "sub-unit" vaccines they are more

difficult to standardize, store and distribute. The advantages and disadvantages of each type of vaccine product are listed in Table 2.

One of the earliest vaccine trials for patients with neuroblastoma used tumor cells combined with bacilli Calmette-Guérin. Subcutaneous injection of this product caused local inflammation and induced transient tumor responses, although the results were never formally published [16]. Subsequently, a second group evaluated DCs loaded with tumor cell lysates. Although an increased number of *in vivo* tumor-specific T cells were detected, no sustained clinical responses were observed [22].

Bowman *et al.* [23] tested the hypothesis that modifying autologous neuroblastoma cells to secrete IL-2 could generate an immunogenic whole-cell vaccine. Local IL-2 secretion recruits T cells and natural killer (NK) cells, and also induces interferon gamma (IFN- γ) release by NK cells and some T lymphocytes [16, 25]. Release of IFN- γ may have significant consequences, since it increases MHC molecule expression by neuroblasts [16] and thereby augments both their immunogenicity and vulnerability to MHC restricted T cell killing. The group found that administration of IL-2 gene-modified autologous tumor cells to patients with high-risk disease was safe and led to a local infiltration of CD3⁺CD4⁺ T cells at the injection site. In the peripheral blood, there were increased numbers of activated T cells, and to a lesser extent NK cells, eosinophils and monocytes. Furthermore, IgG anti-tumor antibodies were identified in 4 of 9 patients. Of the 10 patients treated, 3 had clinical responses after vaccination (1 complete response, 1 partial response, and 1 patient with stable disease) [23]. Additionally, 3 of the remaining 7 patients developed responses (1 complete, 2 partial responses) after low dose oral etoposide [16].

While autologous tumor vaccine products should express all the tumor antigens necessary to generate effective immune responses in a given patient, they are difficult and slow to prepare, and require access to tumor samples from each subject. An allogeneic “off-the-shelf” product is easier to make and standardize, and would broaden the eligible pool of patients in clinical trials. The same group of investigators therefore compared the efficacy of an allogeneic tumor product transduced to express IL-2. Of 12 subjects, 3 had transient increases in the frequency of cytotoxic T lymphocyte precursor cells that reacted to the tumor cell line, but no patients developed cytotoxic immune function against their own tumor cells. Clinically, 1 patient had a >90% partial response after receiving this vaccine [24].

Pre-clinical studies then showed that the T-lymphocyte recruiting chemokine lymphotactin (LTN) [26] could substantially increase the immunogenicity of IL-2 gene modified neuroblastoma cells [27]. This led Rousseau *et al.* [28] to modify the previously used allogeneic cell line to secrete both IL-2 and LTN. Subcutaneous administration to subjects with neuroblastoma led to increased local infiltration of CD4⁺ and CD8⁺ T cells, eosinophils, and Langerhan's cells. Systemically, increased NK cells and IgG antibodies to the vaccine cell line were detected in the peripheral blood. Amongst 28 patients, there were 4 complete responses (2 sustained > 4 years after vaccination), 1 very good partial response, 1 partial response, and 5 patients with stable disease [16, 28] (and unpublished data).

Hence, cell based vaccine therapies for patients with neuroblastoma can be given safely and are able to induce both immune and clinical responses. We and others are now evaluating the safety, immune response, and efficacy of neuroblastoma vaccines given to patients whose disease burden has recently been minimized by high-dose chemotherapy and stem cell rescue. The hope is that vaccination at this time will ensure the best possible ratio of effector cells to tumor cells, and minimize the influence of regulatory T lymphocytes. One of these studies uses an IL-2 and LTN secreting allogeneic cell line injected with a second, unmodified, cell line expressing a distinct set of TAA. This combination of lines should increase the breadth of the resulting immune response, and thereby increase the probability of a response targeting antigens expressed by the patients own tumor.

ADOPTIVE T CELL THERAPY

Adoptive cellular immunotherapies for neuroblastoma are clinically less developed than monoclonal antibodies or vaccines. The technical and regulatory demands of *ex vivo* manufacture and subsequent administration of cellular products are substantial. Nonetheless, there are clear potential advantages in preparing an engineered effector T cell product in an *ex vivo* environment, since it will be free of the immunosuppressive influences of an established tumor. These hypothetical benefits may make adoptive T cell transfer more effective than vaccination, which relies on activation and recruitment of an immune system already damaged both by the tumor and its treatment.

The native T cell receptor recognizes processed protein antigens presented in association with MHC molecules on the cell surface. Thus T cells, unlike MAbs, may recognize processed internal and external antigens, but may be thwarted if the cancer cells have defects in antigen processing/presentation, or fail to express MHC molecules. Despite this limitation, adoptively transferred T cells have several advantages over MAbs. They can expand *in vivo* following transfer and produce long-term re-population of the host with effector cells. They can actively migrate through multiple tissue planes. Lastly, they use direct and indirect cytotoxic effector mechanisms, reducing the risk that tumor cells will be invulnerable to killing.

Prerequisites for generating antigen-specific cytotoxic T lymphocytes (CTL) include the identification of appropriate TAA and the availability of suitable antigen-presenting cells (APCs) [10]. Once identified, antigen-specific CTL lines can be generated by co-culturing T cells with APCs that express the TAA, and expanded by antigen re-stimulation and exposure to cytokines such as IL-2, IL4 and IL7.

Neuroblastoma expresses several antigens of the oncofetal or cancer testis (CTA) group that could be targets for the native receptor of T cells. These include melanoma antigen encoding gene (MAGE)-A1, MAGE-A3/A6, NY-ESO-1, B melanoma antigen (BAGE) and G antigen (GAGE) [29–33]. Although primary neuroblastoma cells express few if any MHC molecules [29, 34], these structures become markedly upregulated following chemotherapy, radiation, or any inflammatory response. Once MHC molecules are expressed, tumor cells may become susceptible to killing by TAA specific T cells. With the increasing successes of

adoptive immunotherapy of T cells directed to oncofetal/CTA, interest has been renewed in attacking neuroblastoma by the same means, but no clinical studies have yet been reported.

As neuroblastoma, like other cancer cells, can downregulate MHC molecule expression [35], there is also interest in the adoptive transfer of T cells expressing an MHC-independent antigen receptor. Such chimeric antigen receptors (CARs) can be generated by linking the heavy and light chain variable regions of a TAA-specific monoclonal antibody. The single chain variable fragment (scFv) is then coupled to the transmembrane and intracellular portion of the ζ -chain signaling moiety from native T cell receptors (Fig. 2) [10, 36, 37]. This construct combines the specificity of monoclonal antibodies with the trafficking and cytotoxic effects of a T cell.

CAR-specific T cells have been used in two clinical trials in patients with neuroblastoma. Gonzalez *et al.* described the generation of CE7R CAR-expressing CD8⁺ clones targeting CD171 [38], the L1 adhesion molecule that is overexpressed by neuroblastoma cells [17, 39, 40]. The CAR-T cells were used in a Phase I trial for patients with recurrent/refractory disease, and of the 6 patients treated, no severe toxicities were observed and one had a partial, but un-sustained, response after CE7R T cell infusion [41].

In the second trial, patients with a history of high-risk neuroblastoma were infused simultaneously with two different T cell populations, activated T cells (ATC), and EBV specific CTL [42]. Earlier adoptive transfer studies had shown that EBV-CTLs are long lived in the peripheral circulation [43–45]. This characteristic is likely attributable to the stimulation and co-stimulation received following native antigen receptor engagement by the EBV antigens chronically expressed by B-lymphocytes in EBV seropositive individuals. The purpose of the study was to discover if long-term survival of CTL continued when the cells also expressed a CAR targeting a neuroblastoma TAA. Prolonged survival of effector cells would be desirable since this would likely increase their anti-tumor activity compared to cells only present transiently.

The subjects in this study received ATC and CTL that both expressed a CAR specific for GD2 (see Monoclonal Antibodies above). However, the CAR used in each cell population contained a unique oligonucleotide that allowed them to be molecularly distinguished [42]. The results showed that GD2-CAR CTL were present longer and at higher levels than CAR ATC, and demonstrated the *in vivo* survival advantage of CTL even when genetically modified to express a CAR. Clinically, no dose limiting toxicities were identified, and in particular no patient suffered neurological pain - a common adverse effect with GD2 MAb and attributable to reaction with GD2 expressed at low levels on peripheral pain fibers. Of 12 patients treated, 3 had no evidence of disease at the time of infusion. One remains disease free at >17 months post infusion, and the other 2 are alive with disease 13 and 34 months post infusion, respectively. Of the 9 patients with relapsed/resistant disease at the time of infusion, 3 have had stable disease (from 10 to 16 months), 2 developed areas of tumor inflammation and necrosis, 1 had clearance of marrow disease at 6 weeks, and 1 has a complete response sustained for more than 24 months [42]. Of note, although patients with neuroblastoma have elevated serum levels of GD2 [11, 46], CAR-CTL function is not

impaired in the presence of their soluble ligand, presumably because of greater avidity of the CAR for antigen on the surface of the tumor cells [10, 47].

While the above approach is encouraging, it has several limitations. Not all patients are EBV seropositive, and in those who are, the manufacture of EBV-CTLs adds to the time, cost and complexity to adoptive cellular immunotherapy. One way to by-pass CTL generation is by modifying CARs to provide additional signals to the T cell following chimeric receptor engagement. T cells require multiple co-stimulatory signals for an optimal response to binding at their antigen specific receptor. These co-stimulatory signals are rarely provided by tumor cells [10], and primary neuroblastoma tumor cells lack most co-stimulatory molecules, including CD40, CD80, CD86, OX40L and 41BB-L [48, 49]. It is possible to modify CARs to express endodomains from these co-stimulatory molecules. CAR binding to the TAA then leads to an array of stimulatory and co-stimulatory signals, and enhanced cell activation, survival and proliferation. Although this approach has worked well *in vitro* [50], it has yet to be assessed *in vivo*.

In the future, other forms of CAR T cell engineering will be evaluated for use in adoptive T cell transfer. Two areas currently being investigated are increasing the tumor-homing capacity of adoptively transferred T cells by incorporating receptors targeting chemokines secreted by tumor cells or cells in the tumor stromal environment [51–53], and modifying the CAR exodomains to reduce the immune response caused by the rodent sequences they contain [10, 38].

CONCLUSION

Immunotherapy using monoclonal antibodies, active immunization or adoptive transfer of antigen specific T cells may provide less-toxic, therapeutic options for patients with neuroblastoma and can produce additional sustained clinical responses. Over the next decade the challenge will be to more firmly establish the effectiveness of these immune approaches and to discover how they may best be integrated with current multimodality treatments.

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Fig. (1). Immunotherapy for neuroblastoma

Currently there are 3 forms of immunotherapy that have been used to treat patients with neuroblastoma: (a) monoclonal antibody therapy, (b) vaccine therapy, and (c) adoptive cell transfer.

Fig. (2). Generation of a chimeric antigen receptor

Chimeric antigen receptors (CARs) consist of an extracellular antigen-specific region and an intracellular chain with kinase activity (endodomain). Upon binding of the target antigen, the endodomain delivers a downstream signal initiating T cell activation.

Table 1

Vaccine Trials in Patients with Neuroblastoma

Vaccine Product Authors	[Reference]
DCs pulsed with tumor lysate	Geiger <i>et al.</i> [22]
DCs pulsed with tumor RNA	Caruso <i>et al.</i> [21]
<i>Autologous</i> tumor cells genetically modified with IL-2	Bowman <i>et al.</i> [23]
<i>Allogeneic</i> tumor cells genetically modified with IL-2	Bowman <i>et al.</i> [24]; Haight <i>et al.</i> [54]
<i>Autologous</i> tumor cells genetically modified with IL-2 and lymphotactin	Russell <i>et al.</i> [55]
<i>Allogeneic</i> tumor cells genetically modified with IL-2 and lymphotactin	Rousseau <i>et al.</i> [28]

Abbreviations: Dendritic cell (DC); Interleukin (IL).

Table 2**Tumor Vaccine Products: Advantages and Disadvantages**

Product	Advantages	Disadvantages
Single peptide	Easy to produce and standardize Immune monitoring of a single antigen	Prior identification of the single TAA Antigen-loss tumor variants
DCs pulsed with peptide, mRNA or apoptotic bodies	Antigen presentation using both MHC class I and II molecules	Complex production Antigen-loss variants Peptide use requires prior identification of the single TAA
Autologous whole cell tumor vaccine	No need to identify TAAs All tumor antigens likely represented and correct MHC restriction insured	Requires patient tumor sample Complex production
Allogeneic whole cell tumor vaccine	No need to identify TAAs Easier to produce and standardize vs. autologous product MHC mismatch may be overcome by cross priming	Inappropriate tumor antigen presentation Correct antigens may be missing

Modified from Rousseau *et al.* 2005 [16].

Abbreviations: Dendritic cell (DC); Major histocompatibility complex (MHC); Tumor associated antigen (TAA).