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1. Introduction

Neuroblastoma, a malignant embryonal tumor of the neural crest cells, is one of the most common solid extracranial tumors of early childhood (Brodeur et al., 2006). The prevalence of neuroblastoma in children is 7.5 cases /100,000 infants (Brodeur et al., 2006; Gao et al, 1997; Gurney et al., 1997; Spix et al., 2006). Furthermore, the annual incidence of this tumor is nearly 1.0/100,000 children under the age of 15 years (Ries et al., 2005). Neuroblastoma comprises about 8-10% of all childhood cancers and for approximately 15% of cancer deaths in children (Gao et al., 1997; Ries et al., 2005). Neuroblastoma can arise anywhere along the sympathetic nervous system. Fifty percent of the tumors originate in the adrenal medulla. Additional sites of origin include the nerve tissue in the chest, neck, pelvis or abdomen (Ries et al., 2005). Clinical presentation of neuroblastoma is highly variable and is dependent on the site of the primary tumor, as well as on the disease extent and on the absence or presence of paraneoplastic syndromes (Maris & Matthay, 1999; Park et al., 2008). The three main clinical scenarios are: localized, metastatic and 4S (S for special).

In contrast to many other pediatric malignancies, progress in treatment of neuroblastoma (especially for advanced-stage tumors) has been relatively modest. Hence, at present, this tumor still poses a major challenge to the pediatric oncologist. Current treatment strategies include any or all of the following; watchful waiting, surgery, mild to aggressive chemotherapy, radiotherapy, and bone marrow transplants. Several new agents and combinations are in ongoing trials for relapsed neuroblastoma, including topoisomerase 1 inhibitors, radionuclides, histone deacetylase and tyrosine kinase inhibitors, monoclonal antibodies directed to distialoganglioside, and angiogenesis inhibitors (Maris & Matthay, 1999). As neuroblastoma is a disease that most often strikes young children, treating patients with aggressive therapy is a concern because of the potential for long-term health implications (from heart disease to second malignancies). Some children with neuroblastoma can be cured, and for these children, oncologists must try to give the minimum treatment possible while achieving cure. Although a fraction of the patients are cured with current treatments, approximately 40 per cent will die of this disease; for these patients improved treatment options are imperative (Matthay et al., 1999, 2009).
2. Virotherapy for cancer

Poor response to conventional treatment is not only observed in neuroblastoma but also in many prevalent tumors. In fact, despite aggressive radio-and chemotherapy, the long-term survival from common cancers such as prostate, breast, colorectal and lung has barely budged since the 1970s (Leaf, 2004). Therefore new cancer treatments with novel mechanisms of action are needed.

Such new treatments may be based on human non-pathogenic and pathogenic viruses (for review see Kirn et al., 2001; Parato et al., 2005; Vähä-Koskela et al., 2007). An oncolytic virus is a replicating agent that has either been naturally selected or engineered to single out and destroy tumor cells. For a long time, viruses have been under scrutiny for their potential antineoplastic effects (Sinkovics and Horvath, 1993). Historically, human trials were initiated with several potential oncolytic viruses during the 1950’ and 1960’s. Among the first viruses to be tested as oncolytic agents were rabies virus (DePace, 1912; Pack, 1950), several adenovirus serotypes (Huebner et al., 1956; Southam et al., 1952), mumps virus (Asada, 1974; Okuno et al., 1978), and West Nile virus (Southam & Moore, 1952). Moreover, 19 different viruses were evaluated in patients suffering from various types of cancer (Newman & Southam, 1954; Wheelock & Dingle, 1964). In general, the outcome of these treatments were tantalizing, but ultimately disappointing and the approach was temporarily abandoned. The onset of the gene therapy era in 1990s reinvigorated the field of viral therapy for cancer (virotherapy). Many of the basic principles of virus-mediated oncolysis apply equally to gene therapy vectors and oncolytic viruses. The main advantage of replication-competent oncolytic viruses over non-replicanting viral gene therapy is the ability to propagate and spread from the site of inoculation throughout tumor mass and beyond. Following the successful attempt to engineer a Herpes virus simplex-1 (HSV-1) mutant that selectively destroyed brain tumor cells (Martuza et al., 1991), the field of virotherapy has expanded considerably. There are now more 90 different DNA and RNA virus variants that have been tested for anti-tumor efficacy in animals and humans (for comprehensive list of oncolytic viruses see Kirn et al., 2001; Parato et al., 2005; Vähä-Koskela et al., 2007). Furthermore, more than 20 different oncolytic viruses have entered clinical trials (for review see Kirn et al., 2001; Parato et al., 2005; Vähä-Koskela et al., 2007).

Oncolytic viruses replicate preferentially in cancer cells because they are taking advantage of the same cellular defects that lead to tumor growth. Viruses that are not inherently selective for cancer cells can often be modified and engineered for tumor-selectivity. Generally, four main approaches have been described in the control of tumor progression through virotherapy: 1) the use of naturally occurring oncolytic viruses; e.g., Newcastle diseases virus (NDV) (Lorence et al., 1988); reovirus (Coffey et al., 1998), and vesicular stomatitis virus (VSV) (Stojdl et al., 2000); 2) engineered viruses with incorporate tissue-specific promoters that limit to tumor cells the expression of genes necessary for replication, e.g., HSV (Miyatake et al., 1997) and adenovirus (Hallenbeck et al., 1999); 3) modification of the viral coat to target uptake specifically to tumor cells, e.g., measles (Hammond et al., 2001); and 4) deletion of entire genes or functional gene regions that are necessary for efficient replication and/or toxicity but are nonessential in cancer cells, e.g., vaccinia virus (Mastrangelo et al., ) and adenovirus (Bischoff et al., 1996). Oncolytic viruses are multimodality therapeutics that can be exploited as a treatment platform for cancer. One advantage of virotherapy is its use in combination with conventional chemo-, radio-or immunotherapy (Aghi & Martuzza, 2005; Kottke et al., 2008; Kumar et al., 2008;
Nandi et al., 2008). Another advantage of oncolytic viruses is the capacity to trigger tumor destruction by different mechanisms including stimulation of innate and adaptive immunity that have the potential anticancer activity (Bell et al., 2003). Despite the impressive improvements made in the field of virotherapy, the use of oncolytic viruses still face important hurdles in cancer therapy. Some of these problems are incomplete transduction, poor systemic distribution, immune response, intratumoral spread, and safety. These issues have to be resolved before oncolytic viral products became approved therapeutics.

3. Oncolytic poliovirus for treatment of neuroblastoma: Preclinical studies

Poliovirus (PV) has recently been added to the list of viruses that hold promise as possible agents in tumor therapy (Gromeier et al., 2000; Ochiai et al., 2004). A nonenveloped, plus-stranded enterovirus of the Picornaviridae family, poliovirus replicates in the gastrointestinal tract causing little, if any, clinical symptoms. Rarely (at a rate of $10^{-2}$ to $10^{-3}$), the virus invades the central nervous system (CNS) where it targets predominantly motor neurons, thereby causing paralysis and even death (Mueller et al., 2005). Generally, poliovirus replicates efficiently in nearly all tumor cell lines tested, which has led to the suggestion that it may be suitable for the treatment of different cancers. However, the possibility that poliovirus can cause poliomyelitis calls for significant neuroattenuation to avoid collateral neurologic complications in cancer treatment. Therefore, the aim of our previous study was to develop highly attenuated polioviruses that may be suitable for the treatment of neuroblastoma in children (Toyoda et al., 2007). It was of concern, however, that the high coverage of antipolio vaccination in early childhood may interfere with the application of poliovirus in tumor therapy. Therefore, we also aimed to develop an immunocompetent animal model that would allow us to investigate the oncolytic capacity of neuroattenuated polioviruses for the treatment of neuroblastoma in the presence of high titers of poliovirus neutralizing antibodies (Toyoda et al., 2007). As shown by us and other investigators, pathogenesis of neurotropic viruses including poliovirus can be controlled by translation (Gromeier et al., 1996, 2000; Mohr, 2005). In poliovirus, an exchange of the internal ribosomal entry site (IRES) within the 5'-nontranslated region (NTR) with its counterpart from human rhinovirus type 2 (HRV2), another picornavirus, yielded viruses [called PV1(RIPO)] that are highly attenuated in mice transgenic for the human poliovirus receptor (PVR) CD155 (CD155tg mice; Gromeier et al., 1996, 1999) yet replicate efficiently and lytically in cell lines derived from malignant glioma and breast cancer (Cello et al., 2008; Gromeier et al., 1996, 2000; Ochiai et al., 2004, 2006). However, PV1(RIPO) and PVS(RIPO), a derivative of PV1(RIPO) that is currently under investigation for the treatment of glioma, grow poorly in neuroblastoma cells (Cello et al., 2008; Gromeier et al., 1996, 2000). This observation prompted us to search for other poliovirus derivatives with oncolytic properties against neuroblastoma. The whole genome synthesis of poliovirus (Cello et al., 2002) produced the surprising observation that a point mutation (A103G) in a "spacer region" between the cloverleaf and IRES in the 5'-NTR that was introduced as genetic marker attenuated poliovirus 10,000-fold (De Jesus et al., 2005). We found that the A103G variant of poliovirus replicates well in human neuroblastoma cell lines at 37°C (De Jesus et al., 2005). However, the attenuating mutation A103G in the spacer region was unstable on replication and direct revertant variants that had acquired the neurovirulent phenotype of wild-type (wt) poliovirus type 1 (Mahoney) [PV1(M)] (Fig. 1A ) were readily scored (De Jesus et al., 2005). We reasoned that a stable attenuation phenotype could be generated if the spacer
region would be interrupted by an essential RNA replication element that the virus cannot afford to delete. Such an element is the cre, a stem-loop structure mapping to the coding region of viral protein 2CATPase (Fig. 1B; Paul, 2002). Cre is absolutely required for poliovirus genome synthesis (Paul, 2002). Based on this concept, we have developed a stable attenuated poliovirus, replicating in neuroblastoma cells, by introducing the cre element into the spacer region between the cloverleaf and IRES in the 5'-NTR (mono-crePV) at the 104-nucleotide locus (Fig 1B) (Toyoda et al., 2007).

Fig. 1. Schematic diagram of full-length poliovirus genomes. (A) Genomic structure of PV1(M). The 5’ end is terminated with the genome-linked protein VPg and the 3’ end with polyadenylic acid. The 5’NTR, harboring the cloverleaf and the IRES, is followed by the single open reading frame (open box) encoding the viral polyprotein, and the 3’NTR. The cis replication element (cre) is indicated as an open stem-loop in the poliovirus polyprotein below polypeptide 2C. The polyprotein contains (Nterminus to C-terminus) structural (P1) and non-structural (P2 and P3) proteins that are released from the polypeptide chain by proteolytic processing. (B) Structure of A133G mono cre PV genomic RNA. The naïve cre in 2C was inactivated through three mutations G4462A C4465U A4472C of the poliovirus-cre loop. The cis replication element (cre) was inserted between cloverleaf and IRES. A point mutation was engineered at nt133 (A to G) of mono-cre PV.

To induce neuroblastoma in our animal model we used a mouse neuroblastoma cell line stably expressing CD155 x (Neuro-2aCD155). Neuro2aCD155 cell line was developed in our laboratory and is susceptible to poliovirus infection (Mueller & Wimmer, 2003). Using the nude mice model, we and others have shown previously that tumors of human origin can be successfully treated with neuroattenuated poliovirus strains, that is with PV(RIPO) derivatives (Groemeier et al., 2000), or with the Sabin vaccine strains (Toyoda et al., 2004). However, in the nude mice the lack of a possible immune response to the oncolytic agents mitigates the importance of the results. Therefore, we constructed fully immunocompetent mice (CD155 tg A/J mice) that express CD155 and accept Neuro-2aCD155 cells for the
formation of lethal neuroblastoma (Toyoda et al., 2007). Using this animal model, we demonstrated that Neuro-2a<sup>CD155</sup> subcutaneous tumors of Neuro-2a<sup>CD155</sup> cells were eliminated by intratumoral administrations of a variant of mono-crePV (A<sub>133</sub>Gmono-crePV) (Toyoda et al., 2007). Interestingly, we observed the complete regression of the established lethal subcutaneous Neuro-2a<sup>CD155</sup> tumors without neurologic side effects despite the presence of high titers of anti-poliovirus antibodies (Toyoda et al., 2007). This result indicates that anti-polio response induced by immunization can exert protection against poliomyelitis without compromising the oncolytic capacity of poliovirus. Remarkably, the tumor-bearing mice, which were cured through treatment with A<sub>133</sub>Gmono-crePV, resisted attempts to reestablish neuroblastoma with Neuro-2a<sup>CD155</sup> cells. We hypothesized that destruction of tumor cells by A<sub>133</sub>Gmono-crePV can increment the release of tumor antigens which may induce a more efficient antigen presentation and the development of a robust antitumor immunity (Toyoda et al., 2007).

Based on these results, we proposed to characterize the antitumor immune response evoked by the treatment of subcutaneous neuroblastoma by A<sub>133</sub>Gmono-crePV. To evaluate the cellular anti-tumor immunity induced by oncolytic therapy with live attenuated poliovirus, we quantified the cytolytic anti-tumor activity of splenocytes collected from the neuroblastoma-implanted CD155 tg A/J mice cured by four intratumoral inoculations of A<sub>133</sub>Gmono-crePV. Mock-treated mice received equivalent intratumoral injections of PBS. These animals were killed after the tumor had reached a volume of ~500 mm<sup>3</sup> and their splenocytes were used as a control in cytotoxic assays. The development of tumor specific cytotoxicity was assessed by standard lactate dehydrogenase-release assays (Decker & Lohmann-Matthes, 1988). Splenocytes isolated from mice cured from neuroblastoma exhibited significantly higher lytic activity against both target cells tested (Neuro-2a<sup>CD155</sup> and Neuro-2a) than did those from splenocytes derived from control mice (16.0% vs 1.5%, P < 0.001). Thus, this result confirmed that treatment with a neuroattenuated oncolytic PV strain induces antitumor immunity against neuroblastoma (Toyoda et al., 2011).

To determine which cell subpopulations are responsible for the cell-mediated antitumor immune responses, splenocytes from the cured mice were depleted in vitro of NK, CD4<sup>+</sup> or CD8<sup>+</sup> cells respectively, prior to cytotoxic assay. As shown in Fig. 2, incubation of splenocytes with neutralizing antibody NK1.1 or anti-CD4 had little or no effect on their ability to kill Neuro-2a<sup>CD155</sup> cells. In contrast, depletion of CD8<sup>+</sup> cells abrogated the cytolytic activity of splenocytes from cured mice by about 70% compared to non-depleted splenocytes (Fig. 2). These data indicate that cytotoxic CD8<sup>+</sup> T cells are the principal mediators of antineuroblastoma immunity elicited by A<sub>133</sub>Gmono-crePV virotherapy (Toyoda et al., 2011)

Any firm conclusion about the capacity of A<sub>133</sub>Gmono-crePV induced antitumor immunity requires direct demonstration of that ability. We thus adoptively transferred the splenocytes harvested from mice cured of neuroblastoma by four A<sub>133</sub>Gmono-crePV inoculations into mice that had developed subcutaneous Neuro-2a<sup>CD155</sup> tumor. Splenocytes from naïve mice served as a negative control. Splenocytes from cured or naïve mice were adoptively transferred by tail vein injection (2x10<sup>7</sup> splenocytes in 100 µl of PBS) to neuroblastoma implanted mice when their subcutaneous tumor volumes were ~ 170mm<sup>3</sup>. After the splenocyte transfer, tumor sizes were measured and tumor volumes calculated every day. Our results showed that adoptive transfer of splenocytes from A<sub>133</sub>GmonocrePV-treated mice produced a significant inhibition of tumor growth by comparison with the negative
control (average tumor volume: 583 mm\(^3\) vs. 2183 mm\(^3\), \(p<0.01\) at day 10 post splenocytes transfer) (Toyoda et al., 2011). This result confirms that oncolytic therapy for neuroblastoma with attenuated poliovirus induces tumor-specific immune response.

![Graph showing % specific lysis](https://www.intechopen.com)

**Fig. 2.** Characterization of effector cytotoxic cells. Mice were sacrificed 2 months after tumor rechallenge. Splenocytes purified from the mice were incubated with neutralizing antibody against CD4, CD8, NK or PBS (as control) and then tested for cytotoxicity against Neuro-2a\(\text{CD155}\) cells.

In line with our findings, previous investigations in mice and humans have also shown that treatment with oncolytic viruses can result in the enhancement of antitumor immune response (Diaz et al., 2007; Greiner et al., 2006; Moehler et al., 2005; Liu et al., 2007a, 2007b; Qiao et al., 2008; Toda et al., 1999).

Tumor destruction by an oncolytic virus can release a wide range of tumor specific antigens that will be taken up by infiltrating antigen-presenting cells for cross-presentation to T cells for priming of antigen-specific immune response (O’Shea, 2005). Moreover, different stimuli can promote an immunogenic cell death of tumor cells. Immunogenic cell death implies that dying tumor cells release several cellular signals that will facilitate immune recognition and elimination of tumor cells (Obeid et al., 2007a, 2007b). Noteworthy, it has been speculated that virus infection of tumor cells may induce an immunogenic cell death (Blachere et al., 2005). Based on these premises, we reasoned that immunization with in vitro PV-infected neuroblastoma cells should be able to prime an effective immune response against tumor cells and subsequently hinder neuroblastoma growth. To this end, 3 freeze-thaw cycles followed by 10 strokes of dounce homogenizer were performed for preparation of noninfected and PV-infected Neuro-2a\(\text{CD155}\) lysates. The poliovirus titer in the PV-infected Neuro-2a\(\text{CD155}\) lysate was \(5 \times 10^8\) pfu/ml. For the tumor rejection assays, polio Neuro-2a\(\text{CD155}\) immunized CD155 tgA/J mice were injected intraperitoneally with PV-infected lysate, or
noninfected Neuro-2aCD155 lysate, or a mixture of noninfected Neuro-2aCD155 lysate plus poliovirus (poliovirus titer in the mixture was 5x 10^8 pfu/ml) or PBS thrice at 1-week interval. All mice were injected intravenously with 2 x 10^6 Neuro-2aCD155 cells 21 days after last vaccination. Survival of the mice was monitored.

Examination of dead mice showed multiple liver and perirenal tumors. Analysis of Kaplan-Meier curves using the log-rank test showed no difference in the survival kinetics among mice immunized with PBS, noninfected Neuro2aCD155 lysate and the mixture of noninfected Neuro-2aCD155 lysate and poliovirus (p> 0.05, Fig 3). All mice from these groups died before 90 days of tumor challenge. In contrast, 70% of mice immunized with PV-infected Neuro-2aCD155 lysate, survived beyond 100 days after tumor challenge. Furthermore, statistical analyses showed that immunization with PV-infected Neuro-2aCD155 lysate was superior to each immunization with other lysate or PBS (p< 0.01, Fig 3).

![Fig. 3. Survival of mice inoculated with Neuro-2aCD155. Before challenge, mice were vaccinated with different lysates or PBS. Mice were observed every day and survival was plotted using a Kaplan-Meier survival curve.](image)

We ruled out the possibility that protection effect seen in mice immunized with PV-infected Neuro-2aCD155 lysate is due to a cytolitic effect by an ongoing poliovirus infection after lysate injection. Our assertion is based in the following facts: i) no poliovirus were isolated from the lung, liver, spleen, brain and spinal cord of mice at the time of tumor challenge, i.e., 21 days after vaccination with homogenates prepared from PV1(M)-infected Neuro-2aCD155 cells or noninfected Neuro-2aCD155 admixed with poliovirus, ii) No protection was induced by noninfected Neuro-2aCD155 admixed with poliovirus, and iii) all mice are vaccinated with different lysates or previously immunized against poliovirus. Altogether,
our results clearly suggest that in vitro infection of neuroblastoma cells turn these cells into a potent tumor immunogen.

It has long been assumed that most therapeutic agents kill tumor cells through apoptosis. Notably, apoptosis has been considered to be a non-immunogenic or even a tolerogenic cell death mechanism as opposed to necrosis (an immunogenic cell death). However, depending on the cell death inducer, tumor cells can become highly immunogenic and induce a potent antitumor response in vivo (Zitvogel et al., 2006). Furthermore, there is now persuasive evidence that cell death can trigger an immune response only if the dying cells emit ‘eat-me’, ‘danger’ and ‘killing’ signals that mediate their efficient phagocytosis by dendritic cells (DC’s) and the maturation of DCs (Blachere et al., 2005; Casares et al., 2005; Kepp et al., 2009; Melcher et al., 1998; Sauter et al., 2000).

DCs are the most important professional antigen-presenting cells and play a central role in initiating innate and adaptive immune response against tumor associate antigens (TAA). Antigen presentation by immature DCs (iDCs), associated with a lack of costimulation, induces tolerance. A number of molecules released from dying cells have been identified to elicit immune signaling during immunogenic cell death. These include, among others, high mobility group box 1 (HMGB1, also known as amphoterin), purine metabolites (uric acid), calreticulin (CRT), and heat shock proteins (HSP) (Freedman et al., 1988; Obeid et al., 2007a; Shi et al., 2003; Udono & Srivastava, 1994). Induction of HSP is one of the earliest indications of cellular stress following infection with both RNA and DNA viruses (Asea, 2007; Liu et al., 2008). HSPs have been found to play key roles in the stimulation of the immune system when located on the plasma membrane or in the extracellular space (Udono & Srivastava, 1994). These proteins can promote tumor antigen cross-priming by binding to DC (Schild et al., 1999; Srivastava et al., 1998).

Moreover, HSP 70 and 90 expressions act as ‘eat me’ signals and enhance phagocytosis and maturation of DC (Binder & Srivastava, 2004; Lehner et al., 2004). CRT, another ‘eat me’ signal, is located in the lumen of the endoplasmic reticulum (ER) (Obeid et al., 2007a). Upon stress, CRT is translocated and exposed on the outer leaflet of cells during the early phase of cell death (Obeid et al., 2007a, 2007b). The exposure of CRT dictates the immunogenicity of tumor cells death because exposed CRT help the engulfment of dying tumor cells by DC (Obeid et al., 2007a). Investigators have previously identified uric acid as a novel endogenous danger signal capable of alerting the immune system (Shi et al., 2003). Injury cells rapidly degrade their DNA and RNA and release uric acid that activates DCs. Another signal molecule is HMGB1, which is loosely bound to chromatin and is referred to as ‘danger signal’ or alarmin (27). Dying cells release this molecule massively in the extracellular environment (Scaffidi et al., 2002). Extracellular HGMB1 induces functional maturation of DCs and acts as immune adjuvant for soluble and particulate antigens (Dumitriu et al., 2005; Rovere-Querini et al., 2004).

Until now, most of the identified inducers of immunogenic cell death of tumors cells are chemotherapeutics (Ulrich et al., 2008). The potent antitumor response elicited by virotherapy, observed by us and other investigators, clearly suggests that viruses are in vivo inducers of immunogenic death of tumor cells. Moreover, our finding that inoculation of PV-infected neuroblastoma lysate protects against live tumor challenge indicates that poliovirus is also an in vitro inducer of immunogenic death. Interestingly, necrosis by freeze-thawing is also considered as an immunological cell death (Ulrich et al., 2008).

However, we observed that immunization with freeze-thawed noninfected neuroblastoma
lysate, as opposed to the PV-infected lysate, did not protect against live tumor challenge. This result underscores the capacity of poliovirus to trigger the release of danger signals from tumor cells, which in turn it could induce an antitumor response. Significantly, cells infected with encephalomyocarditis virus, a picornavirus as polio, have been shown to be effective at presenting nonviral antigens for cross-priming of DCs in vivo (Schulz et al., 2005). Viral lysates (called oncolysate) have been used in the past to vaccinate human against cancer (Sinkovics & Horvth, 2006). Those few trials have shown some degrees of success, but results have been ambiguous (Cassel & Murray, 1992; Freedman et al., 1988; Murray et al., 1977).

Remarkably, treatment with viral oncolysates showed better overall survival than radio-and chemo-therapy, probably because of the harmful effects of conventional therapies has on the immune system (White et al., 2002). On the other hand, using live viruses in humans for the treatment of cancer comes with some drawbacks. Inoculation of a virus, mainly a high dose, could elicit an unwanted inflammatory reaction, or cause an opportunistic infection or an unexpected complication (Bell et al., 2003; Kirn et al., 2001). Furthermore, the majority of human population is immune to several of the potential therapy viruses (Bell et al., 2003). Therefore, innate and pre-existing immunity may inactive the oncolytic virus which limits its use in systemic virotherapy (Ikeda et al., 1999; Kirn et al., 2001). Tumor vaccine therapy using virus lysate may overcome some of these hurdles. Viral oncolysate can be prepared with nonpathogenic virus without losing its tumor immunogenicity. Hypothetically, inactivation of the live virus present in the viral oncolysate should not affect the antitumor response induced by viral oncolysate. Finally, immunotherapy with viral oncolysate can be more effective than tumor virotherapy in controlling minimal residual and metastatic diseases states, thereby preventing or prolonging the time of recurrence.

4. Hypothesis and future studies

We hypothesize that in vitro poliovirus infection of neuroblastoma cells induces an immunogenic tumor cell death through a massive upregulation of endogenous alarm signals. We entertain the idea that the main alarm signals induced by poliovirus infection of tumor cells are CRT, HSP 70 and 90, uric acid and HMGB1, which in turn mediate DC activation. Therefore, we also hypothesize that PV-infected neuroblastoma lysate induces an effective DC maturation and tumor antigen cross-presentation. Finally, we postulate that immunization with PV-infected neuroblastoma lysate generates a systemic and potent anti tumor response that will eradicate established tumors and will confer a long-lasting tumor immunity.

Overall, there is enough information to expect that the immunization with polio lysate might represent a new treatment for neuroblastoma. However, the antitumor response induced by polio oncolysate remains largely uncharacterized. Therefore, a detailed knowledge of the immune response and therapeutic protection elicited by polio-infected neuroblastoma lysates is needed. Specifically, future studies should aim to determine:

- **Aim I.** If poliovirus infection of neuroblastoma cells induces upregulation, translocation and release of endogenous danger signals.
- **Aim II.** If polio-infected neuroblastoma lysates can induce DC maturation and presentation of tumor-derived antigens
- **Aim III.** Whether immunization with polio-infected neuroblastoma lysates can induce a systemic immune response capable of eradicating established cancer and stimulating a long-lasting anti-tumor immunity
Aim I

Rationale

Tumors evoke mechanisms to induce immune tolerance. In this context, tumor cells are nonimmunogenic and their death will not stimulate an antitumor response. On the other hand, tumor cells can be stressed by multiple stimuli and may promote membrane expression or release of endogenous danger signals. The upregulation of these signals will turn nonimmunogenic tumor cells into immunogenic tumor cells, and facilitate immune recognition and final elimination of the stressed tumor cells. It has been shown that chemotherapeutic agents activate pro-apoptotic promoter BAX, which in turn induce translocation and exposure of two potent danger signals, CRT and HMGB1 (Kepp et al., 2009; Krynetskaia et al., 2008). Interestingly, poliovirus infection activates BAX in neuroblastoma cells (Autret et al., 2007). It is tempting to speculate that different inducers of immunogenic cell death activate common pathways to upregulate, translocate and release endogenous warning molecules. Thus, we postulate that poliovirus infection of neuroblastoma cells induce the upregulation and translocation of CRT, uric acid, HSP 70 and 90, and HMGB1. We also hypothesize that signal-inducing capacity of poliovirus depends on virus strain and infectious dose.

Approach.

Neuroblastoma cells will be infected with different poliovirus strains at different multiplicity of infection (MOI). At different time points after infection, the presence of CRT, uric acid, HSP 70 and 90, and HMGB1 in virus-infected cells and/or their supernatants will be determined. It also important to determine apoptosis/death of infected cells by Annexin V/PI test.

Expected results and alternatives.

Based on our previous results (see above in point 3), we think that poliovirus is a potent inducer of immunogenic cell death. Therefore, it is expected that infection of neuroblastoma cells will lead to surface exposure of CRT, translocation of HMGB1 from nucleus to cytoplasm of infected cells followed by release into cell culture medium. We also anticipate a rapid increase in the expression of inducible HSP70 and HSP 90 and in the concentration of uric acid within cells and in supernatant of polio-infected cells.

With this approach, we should be able to determine the kinetic of different danger signal expressions induced by poliovirus before overt CPE is developed. We should also be capable of correlating the kinetic of danger signals and apoptotic or necrotic profile of the virus-infected tumor cells. Finally, we speculate that there are differences between different poliovirus strains in their capacity to induce upregulation of danger signals, and with our experimental approach we should be able to determine these differences. It is possible that the endogenous danger signals that we are going to analyze are poorly or not at all induced by poliovirus. In this case, we will look for other potential immunogenic determinants of dying tumor cells such as NKG2D ligands, RNA, HSP27, DNA, PTX3, IL-1β. Alternatively, proteomic analyses can be done to identify new putative “alarming” signals induced by poliovirus infection.

The way in which exogenous insults kill a tumor cell is likely to be a key determinant of the interaction of dying cells with the immune system and whether this interaction will lead to an immune response. Here we will define if poliovirus infection of neuroblastoma cells induces an immunogenic cell death through upregulation and translocation of endogenous danger signals. cell death through upregulation and translocation of endogenous danger signals.
Aim II

Rationale

In response to various cell death associated stimuli, Dcs have been shown to play a central role in the recognition of apoptotic cell death and in the initiation of an immune response. Only when danger signals are correctly emitted by dying cells, in response to a stimulus, and perceived by DC, an immune response is elicited. On the other hand, pathogen induced cell death can be immunogenic and hence stimulate an immune response against antigens that derive from dying cells and are presented by dendritic cells (DCs). Here we postulate that poliovirus infection of neuroblastoma cells induces an immunogenic death of these tumor cells. Therefore, lysate from these cells contain several immunogenic (danger) signals and tumor associated antigens that will induce DC maturation and tumor antigen-presentation.

Approach

Initially, mouse DC will be treated with polio oncolysates to determine whether these oncolysate could stimulate DC maturation. To assess DC activation by different viral lysates, phenotypic DC maturation and production of cytokines will be measured. To address whether the DC response to viral lysate could impact on downstream pathways of anti-tumor immune response, we will test stimulation of splenocytes from neuroblastoma-challenged mice via presentation by viral lysate-activated DC. As markers of splenocytes activation, IL 2, 4, 10 and IFN-γ release and cytotoxic activity will be determined.

Expected results and alternatives. As mentioned in Aim 1, poliovirus infection of neuroblastoma cells provokes an immunogenic cell death. Therefore, we expect that the interaction between polio-infected neuroblastoma lysate and iDCs will result in DC maturation, allowing the DCs to activate relevant cell mediated immunity against tumor cells. Our experimental approach will allow us to determine the ability of viral oncolysate pulsed DC to present tumor and viral antigens to primed T cells. Moreover, we expect that oncolysate-pulsed DCs will be able to prime naive splenocytes against tumor antigens. In line with our theory, we speculate that wild type poliovirus is a stronger inducer of immunogenic cell death than the other strains used in this study. Thus, PV1(M)-oncolysate will induce a stronger activation of DC than those stimulated by the polio replicon or the neuroattenuated polio oncolysate. However, we think that DCs will mature and acquire the ability of presenting tumor antigens upon stimulation with polio replicon oncolysate or the neuroattenuated polio oncolysate.

It might be possible that the activation signals provided by oncolysate induce a poor maturation of DCs. To solve this problem, we can concentrate the oncolysates and test their capacity to induce DC maturation. Alternatively, we can mix oncolysate with small amounts of LPS (10-50 pg) to boost DC maturation. It is expected that polio oncolysate has a high concentration of poliovirus proteins. Therefore, DCs may preferentially present poliovirus antigens. In this case, oncolysate-pulsed DC will induce a poor antitumor response. We propose to increase the amount of tumor antigens presented to DCs. For this purpose, we will use cell membrane isolated from neuroblastoma cells as source of tumor antigens. After concentration, cell membranes will be mixed with oncolysate and the mixture will be used to pulse DCs.
Aim III

Rationale

Unfortunately, the prognosis for many children diagnosed with neuroblastoma is poor despite aggressive surgical resection and simultaneous radiochemotherapy regimens. Moreover, the frequency of relapse and the subsequent failure of further treatment have created the need to develop non-toxic and more effective treatments. Immunotherapy has the theoretical appeal that tumor-reactive lymphocyte may seek and eliminate tumor cells with greater accuracy than conventional therapy. A limiting impediment to successful immunotherapeutic treatment is the stimulation of adequate tumor antigen-specific effector cells. To attain this, tumor-associated antigens should be processed by antigen presenting cells (APCs), and presented to T cells along with enough costimulatory signals to avoid tolerance. Our previous results showed that polio oncolysate is able to prime an anti-neuroblastoma response. This result indicates that viral oncolysate contain tumor associated antigens and immunoadjuvant molecules which activate the immune system to elicit a prophylactic tumor response. Based on this finding, we propose to continue our studies and determine if immunization with polio-infected neuroblastoma oncolysate could evoke a tumor-specific immunity capable of eradicating established neuroblastoma as well as maintaining immunological memory. On the other hand, viral oncolysate contain infectious virus. Therefore, inoculation of viral oncolysate may cause severe complications. To circumvent this problem, we also propose to obtain viral oncolysate from neuroblastoma cells infected with polio replicon. These RNA-based vectors are generated by providing the capsid proteins in trans, they can undergo only a single round of replication in the infected cell and are genetically incapable of producing infectious virus. Therefore, we will also determine the capacity of noninfectious poliovirus replicon-infected lysate to induce a therapeutic effect against established tumor.

Approach.

We will establish hepatic and subcutaneous Neuro-2aCD155 tumors in polio-immunized CD155 tg A/J. Ten days after tumor cell inoculation, animals will be vaccinated intraperitonally (i.p) or intramuscularly (i.m) with neuroblastoma lysates prepared from tumor cells infected with wild type PV1 (M), neuroattenuated A133GmonocrepPV or propagation-defective poliovirus (poliovirus replicon). Control mice will be inoculated with noninfected neuroblastoma lysates or PBS. To determine long-lasting anti tumor immunity evoked by oncolysate vaccination, mice previously cured with any experimental treatment described above or 6 months after immunization with different lysates will be challenged i.v. or s.c with neuroblastoma cells. Protection response to each experimental therapy will be measured by survival rates and tumor growth. Splenocytes and sera from each experimental group will be obtained to test tumor-and virus specific immunity.

Expected results and alternatives. First, we expect to obtain a comprehensive characterization of tumor-specific and virus-specific induced by in vivo vaccination with poliovirus oncolysate. We think that in vivo administration of polio oncolysates will mediate a strong therapeutic benefit and eliminate or slow significantly tumor growth, preferentially on established liver metastases. Based on our previous results, we anticipate that in vivo vaccination with polio oncolysate will induce a robust memory response. Since tumor immunogenicity of polio oncolysates might be different depending on poliovirus strain used for their preparation, we expect to see differences in the therapeutic effect induced by each of the oncolysate tested. There is concern that immunity induced by
repetitive inoculation of viral oncolysate will be biased towards a viral immune response. Our experimental approach will allow us to determine the tumor-specific and virus-specific and therapeutic effect induce by in vivo immunization with oncolysate. Correlation of these data should identify whether the biased response affects the effectiveness of immunotherapeutic treatment.

It is possible, although unlikely, that in vivo vaccination with oncolysate will induce a poor anti-tumor response. If we detect that the reason of this poor anti-tumor response is due to oncolysate vaccination bias the immune response to virus, we will increase the tumor antigens in the oncolysate as described in Aim 2. Another possibility for a poor anti-tumor response is a low adjuvanticity of viral oncolysate in vivo. In this case, we propose to mix viral oncolysate with CpG oligodeoxynucleotides. CpG has been successful used as an adjuvant in other mouse models of immunotherapy for treatment of several tumors. Alternatively, we may improve the immune response against the tumor by combining frequency, timing, dose and route of administration of viral oncolysate.

5. Conclusion

In the current state of the art, it is likely that virotherapy using attenuated poliovirus will be capable of eradicating neuroblastoma when used in combination with other therapies. Moreover, immunotherapy with PV oncolysate alone or in combination with virotherapy can be effective in controlling minimal residual and metastatic diseases states, thereby preventing recurrence or prolonging the time of recurrence in patient suffering from neuroblastoma. Therefore, we think that our data and the directions of future studies presented here will lead to practical applications of attenuated poliovirus and polio oncolysate for the treatment of neuroblastoma.

6. References


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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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