Cancer Letters ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form 24 November 2015
Accepted 27 November 2015

Keywords:
Neuroblastoma
Oncolytic virotherapy
Mesenchymal stem cells
Immune response
Cell migration

ABSTRACT

We report here our clinical experience of a program of compassionate use of Celyvir – autologous marrow-derived mesenchymal stem cells (MSCs) carrying an oncolytic adenovirus – for treating children with advanced metastatic neuroblastoma. Children received weekly doses of Celyvir with no concomitant treatments. The tolerance was excellent, with very mild and self-limited viral-related symptoms. Patients could be distinguished based on their response to therapy: those who had a clinical response (either complete, partial or stabilization) and those who did not respond. We found differences between patients who responded versus those who did not when analyzing their respective MSCs, at the expression levels of adhesion molecules (CCR1, CXCR1 and CXCR4) and in migration capacities in transwell assays, and in immune-related molecules (IFNγ, HLA-DR). These results suggest interpatient differences in the homing and immune modulation capacities of the therapy administered. In addition, the pretherapy immune T cell status and the T effector response were markedly different between responders and non-responders. We conclude that multidoses of Celyvir have an excellent safety profile in children with metastatic neuroblastoma. Intrinsic patients' and MSCs' factors appear to be related to clinical outcome.

Introduction

Oncolytic virotherapy is gaining interest in the clinic as a new weapon against cancer [1]. Various clinical trials have been conducted in adults and to a lesser extent also in pediatric patients [2]. Initial results have given information on the toxicities [3] and mechanism of action of this new strategy [4]. Different wild type or genetically engineered viral strains have been used, injected either systemically or intratumorally into patients with advanced or non-curable cancers. Oncolysis is not the only benefit that virotherapy can deliver; it is now well accepted that an important aspect of oncolytic viruses is the antitumor immune response they can initiate or reactivate within the patients, which translates into clinical responses [1]. The role of the immune system during virotherapies has a second edge though. The antiviral immune response of the patients is a major threat and limits the effects of oncolytic viruses [5,6]. The antiviral response may minimize the amount of viruses that eventually reach tumor sites upon systemic delivery, while increasing the doses may only cause higher toxicity and further sensitization toward the oncovirus.

We reported an initial clinical experience in the use of CELYVIR in 4 children with advanced neuroblastoma (NB) [7], the most frequent extracranial solid tumor in pediatric patients. Children with metastatic NB who relapse or develop refractory disease have a dismal prognosis; therefore, new strategies are needed in order to improve the outcome of these patients. CELYVIR is the acronym for autologous mesenchymal stem cells (MSCs) infected with ICOVIR-5, an oncolytic adenovirus [8] designed for systemic treatment of disseminated solid tumors. ICOVIR-5 contains several modifications that give it selective replication ability in cancer cells in which

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http://dx.doi.org/10.1016/j.canlet.2015.11.036
0304-3835/

Please cite this article in press as: Gustavo J. Melen, et al., Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.11.036

the Rb/E2F route is activated [9]. Our strategy consists of systemic infusions of CELYVIR aiming at enhancing the targeted delivery of the oncolytic adenoviruses to the metastases based in the natural tumor tropism of the MSCs. Here, we now report the complete program of compassionate use of this new antitumor medicine, after treating 12 additional children. We have gathered information that confirms the safety of this procedure, enabling numerous infusions per child, amounting to very high doses of virus with very low toxicities. We have also found some clinical responses in our cohort. Patients who responded to treatment showed interesting differences in immunity, before and during treatment, compared to children without response. In addition, their MSCs showed differences in the expression of cell adhesion molecules and immune-related genes when comparing both groups of patients.

Patients and methods

Patients

Twelve patients diagnosed with refractory neuroblastoma were enrolled in a program of compassionate use. The local Research Ethics Board and the Spanish Medicine Agency (AEMPS) approved each patient's treatment in an individualized basis, and informed consent was obtained from each participant. Table 1 shows patients' and infusions' characteristics. Toxicities were studied, recording clinical symptoms and signs of adverse effects, and by hematological and biochemical analysis done in blood samples prior to each infusion. Clinical responses were evaluated after the 6th dose with the level of serum enolase and with ^{123}I MIBG-scintigraphy, comparing the number of lesions pre- and post-therapy.

Cell culture

Bone marrow MSCs were obtained from the iliac crest of patients as previously described [7]. Briefly, mononuclear cells were obtained by Ficoll gradient centrifugation (400 *g*, 25 min, 20 °C) and cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin–streptomycin (P/S, Gibco). The medium was replaced after 48 hours. Cells were maintained at 37 °C and 5% CO_2. MSC production complied with the principles of Good Manufacturing Practice (GMP) in an AEMPS-approved clean room.

For CELYVIR preparation, cell cultures were washed with PBS, trypsinized (TripLE Express, Life Technologies, Carlsbad, CA) and MSCs resuspended in 25 mL of complete medium. MSCS received 30 Gy irradiation, fulfilling a request of AEMPS. 3×10^6 cells/mL cells were then infected with ICOVIR-5 [8] at 200 MOI during 120 min at 37 °C, 5% CO_2 in DMEM. Infected cells were then washed and resuspended in 50 mL of 0.9% saline supplemented with 2% human albumin.

Flow cytometry analysis

Flow cytometry analysis was performed with FACSCanto II and FACSDiva software v6.1.2 (BD Biosciences, Franklin Lakes, NJ). Data acquisition and analyses of cell adhesion and immune-related molecules were performed on the CELYVIR product, i.e., after MSCs were irradiated and infected with ICOVIR-5. The mean fluorescence intensity (MFI) was obtained for each adhesion molecule. Expression levels were normalized to those of their respective isotype control to allow comparisons. The list of molecules studied is available as Supplementary material.

Peripheral blood leukocyte subtypes were studied as previously described by our group [10]. Fresh tumor biopsies were mechanically disaggregated, filtered through a nylon mesh cell strainer, 100 µm (BD Bioscience), and cell suspension was processed for flow cytometry. Dead cells were excluded by 7-AAD staining.

Histological studies

Tumor biopsy specimens from patient UPN5 were fixed in 10% neutral formalin. Paraffin-embedded sections were cut at 3 µm and stained for hematoxylin–eosin. Several antibodies were used for staining tumor infiltrating human leukocytes (see Supplementary Fig. S4). An automated staining system (Dako Autostainer, DakoCytomation, Denmark) was used in combination with a two-step peroxidase-labeled polymer system (Envision System, Dako, Denmark).

Cell migration assays

NB1691 cells (kindly provided by Dr. A. Davidoff of St. Jude's Children's Research Hospital) were seeded at 25,000 cells/cm^2 in the lower chamber of a transwell multiwell plate (BD Biosciences, Franklin Lakes, NJ), in 1% P/S DMEM, and 20,000 Celyvir-MSCs were seeded on the gelatine-coated upper chamber. After 24 h at 37 °C, 5% CO_2, transwells were washed and non-migrated cells were removed with a cotton swab previously dipped in PBS. Migrated cells were fixed in 10% formalin for 20 minutes and stained with 0.2% crystal violet in 10% formaldehyde for 15 minutes. Finally, transwells were washed twice with water and allowed to dry. Each sample was tested in triplicate.

For specific chemokine assays, 10 ng/mL of either CXCL12 or CCL5 was tested following the same scheme as for NB1691. Monoclonal antibodies against these two molecules (200 ng/mL) or monoclonal antibodies against their counterpart receptors (CXCR4 and CCR1,

Table 1
Patients' characteristics.

ID	Gender	Lines TH	Celyvir doses	Cells (×10^6)	Virus	PCR PB	Outcome
UPN5	F	3	70	2640	2.6E+14	+	SD
UPN6a	M	3	6	226	4.5E+12		CR
UPN6b		5	29	1013	2.3E+13	+	PR
UPN7	F	4	9	520	5.2E+13	ND	PD
UPN10	M	4	6	160	1.6E+13	ND	PD
UPN11	M	4	4	150	1.5E+13	ND	PD
UPN12	F	4	10	340	3.4E+13	ND	PD
UPN13	M	4	8	280	2.8E+13	ND	PR
UPN16	F	3	7	150	1.5E+13	+	PD
UPN18	F	4	14	300	3.0E+13	+	PD
UPN19	M	4	9	220	2.2E+13	+	PR
UPN20	F	3	6	290	2.9E+13	+	PD
UPN21	M	6	8	176	1.7E+13	+	PD

ID, identification; TH, therapy; PCR PB, detection of adenoviral genome in peripheral blood by PCR; UPN, unique patient number; SD, stable disease; PR, partial response; CR, complete response; PD, progressive disease; ND, not done.

Please cite this article in press as: Gustavo J. Melen, et al., Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.11.036