Changes in tumor infiltrating leukocytes in neuroblastoma treated with oncolytic virotherapy: insights from preclinical models and patients

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INTRODUCTION

The prognosis of metastatic or relapsed NB is very poor, practically incurable. New therapeutic strategies for increasing cure rates are clearly needed. Our group has been developing a strategy for the treatment of refractory and metastatic childhood solid tumors, based on the administration of *Celyvir*: autologous mesenchymal cells that carry inside the oncolytic adenovirus ICOVIR-5 (EudraCT2008-000364-16; ClinicalTrials Identifier: NCT01844661). The possibility that oncolytic virotherapy stimulates antitumor immune responses opens new possibilities in the field of cancer immunotherapy. Preexisting lymphocytic infiltration in tumors (TILs) is associated with better prognosis in a variety of cancers. Recent studies also indicate that lymphocyte responses can identify those patients most likely to benefit from immune targeted therapies, suggesting that the effectiveness of immunotherapy can be improved through strategies that induce inflammation of the tumor. In our clinical experience we have found that the local and systemic administration of Celyvir caused changes within TILs, associated with a beneficial clinical response.

The main objective of this project is to characterize *Celyvir*-induced changes in TILs and tumor stroma using a murine NB model (which recapitulates the main genetic and clinical aspects of NB with amplified MYCN) and a murine *Celyvir* therapy similar to human.

METHODOLOGY

Human Celyvir

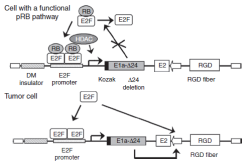
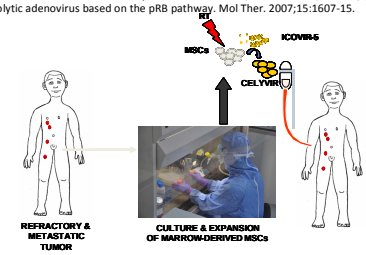
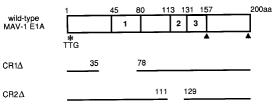


Diagram of the components of ICOVIR-5 that confer selective replication. E1a-Δ24 is unable to disrupt the pRB–E2F complexes, avoiding a positive-feedback loop if the promoter repression by pRB–E2F complexes leaks. Deregulation of the pRB pathway in tumor cells releases free E2F that activate the E2F responsive elements in ICOVIR-5. The presence of the Kozak sequence increases the efficacy of E1a-Δ24 expression. The RGD-modified fiber increases the virus infectivity. Cascallo M et al. Systemic toxicity-efficacy profile of ICOVIR-5, a potent and selective oncolytic adenovirus based on the pRB pathway. Mol Ther. 2007;15:1607-15.

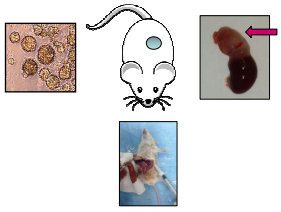


Bone marrow mesenchymal stem cells (MSCs) were obtained from the iliac crest of patients. MSCs production complied with the principles of Good Manufacturing Practice. MSCS received 30 Gy irradiation, were then infected with ICOVIR-5, washed and resuspended in saline supplemented with human albumin, and infused through a central line.

Murine Celyvir



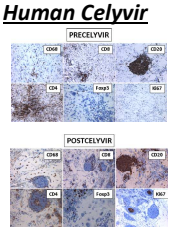
Mouse adenovirus type 1 (MAV-1) E1A protein contains three conserved regions (CR1, CR2 and CR3) that are comparable to the conserved regions found in Ad5 E1A, which interacts with members of the pRB family. MAV-1 *dl102* contains a CR2 deletion which confers oncolytic properties, similar to the Δ24 oncolytic Ad5 vector (Smith K et al. Interaction of Mouse Adenovirus Type 1 Early Region 1A Protein with Cellular Proteins pRb and p107. Virology 1996;224:184-197). MAV-1 dl102 virus was shown to replicate and to have a potent anti-tumor activity in a panel of murine tumor cell lines.



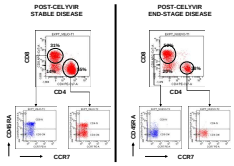
NB neurospheres derived from TH-MYCN (129/SVJ) mice were cultured in DMEM:F12 (50%), 1xB27 (w/o VitA), 40ng/mL of mFGF and 20ng/mL of mEGF. At day 0, 10^6 tumor cells were orthotopically implanted on the adrenal gland of WT 129/SVJ mice. At week 2 and 3, mice were either treated with murine *Celyvir* (mMSC infected with MAV-1 *dl102*) or untreated. Similary to human Celyvir, we infected adipocyte derived mMSC with MAV-1 dl102 oncolytic vector at a MOI of 200 during 90 min. After infection, cells were washed and prepared to treat mice, and mCelyvir was I.V. injected. At week 4, mice were sacrified and tumors were analyzed by flow cytometry (leukocyte infiltration) or qRT–PCR (genes of tumor microenvironment).

RESULTS

Human Celyvir

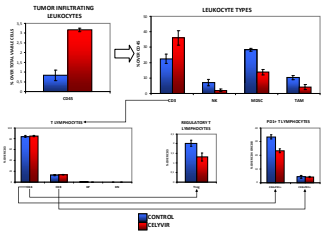


Histological analysis of the tumor of a patient treated with CELYVIR. Biopsies of the primary tumor before and after CELYVIR therapy in a patient with NB. Immunohistochemistry studies in paraffin-embedded biopsies showed the presence of different populations of tumor infiltrating lymphocytes (TILs), pre and post CELYVIR therapy. Interestingly, Ki67 staining showed an increase in the activity of TILs within nodular structures in the tumor mass after CELYVIR therapy.

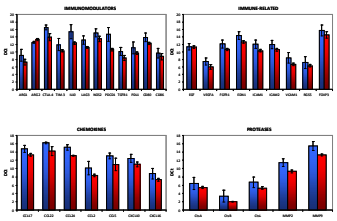


Changes in the phenotype of tumor infiltrating T lymphocytes of a patient after therapy. Flow cytometry of tumor biopsies obtained at 2 different moments during CELYVIR therapy (stable disease and end-stage disease) showed notable changes in the CD4:CD8 ratio and naïve:central memory:effector immunophenotypes of TILs.

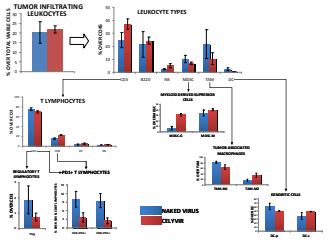
Murine Celyvir



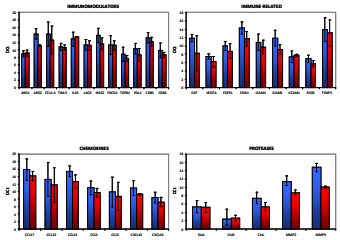
EXPERIMENT #1. Systemic administration of Celyvir provoked changes in local immune tumor infiltration. Mice implanted with NB cells were treated with Celyvir IV, as explained above. Our results (mean ± mean standard error) showed that mCelyvir-treated mice have higher percentage of immune cell infiltration compared to non-treated mice ($p<0,05$), especially T lymphocytes, which suggest that mCelyvir treatment stimulates the recruitment of these immune cells into the tumor microenvironment. On the other hand, levels of myeloid subpopulations tended to be lower in mCelyvir-treated mice compared to non-treated mice. Myeloid activation is classically associated with immune suppression in the tumor microenvironment, so these results might suggest that immune response is being activated in the tumor microenvironment due to the treatment with mCelyvir.



EXPERIMENT #1. Systemic administration of Celyvir provoked changes in local tumor environment. Gene expression assays were performed for genes with known role in cancer immunosurveillance and tumor progression. We found that PD1, MMP2 and CCL24 showed statically significant differences between treated and non-treated mice ($p<0,05$), being these levels lower in mCelyvir-treated mice. In other genes (CTLA-4, ARG2, IL10, LAG3, CCL17, EDN1, ICAM1 and MMP9) we also found a trend to decrease after mCelyvir treatment, but differences were not statically significant due to low number of mice analyzed. It is important to note that CTLA-4 and PD1 expression were diminished in treated mice, suggesting an increased T-cell activation after treatment with mCelyvir associated to changes within the tumor microenvironment.



EXPERIMENT #2. MSCs do not only act as carrier cells but modulate immune tumor infiltration. Mice implanted with NB cells were treated intratumorally with a single dose of either the oncolytic adenovirus or mCelyvir. The percentages of immune cell infiltration were similar comparing both groups of mice, but the subtypes of infiltrating leukocytes differed. The presence of MSCs in the medicine resulted in higher lymphocyte and lower myeloid cell infiltration of tumors. Differences were also detected in subpopulations of T lymphocytes, PD1+ T lymphocytes, myeloid-derived supressor cells, tumor associated macrophages and dendritic cells.



EXPERIMENT #2. MSCs do not only act as carrier cells but modulate local tumor environment. Gene expression levels of genes related with the tumor microenvironment tended to be lower in mCelyvir-treated mice compared to naked virus-treated ones, especially ARG2, CXCL10, CtsL, MMP2 and MMP9, but differences were not statically significant due to low number of mice analyzed.

CONCLUSIONS

1.Systemic administrations of Celyvir induced changes at local tumor sites. These changes affected both the infiltration by immunecompetent cells (lymphoid and myeloid cells, effectors and regulators) and the tumor-tolerant microenvironment. The cellular component of our strategy functions not only as carrier for the oncolytic virotherapy but has a role in the immune responses taking place after CELYVIR infusions. Works are in progress in order to functionally characterize the changes in the tumor infiltrating immune cells and in the tumor-tolerant environment.

2.The immunocompetent animal model and the conditionally-replicative MAV-1 *dl102* should allow us to dissect crucial aspects of the mechanism of action of *Celyvir*, helping in optimizing this strategy in the clinic setting.

Acknowledgments: We thank to Jesús Martinez and Edilia Almeida for animal care.

This work was supported by grants from Fondo de Investigaciones Sanitarias-FEDER PI13/02487, and the Madrid Regional Government (S-BIO-0204-2006, MesenCAM; P2010/BMD-2420, CellCAM). MR is supported by Asociación Pablo Ugarte and by Asociación NEN..