

PHENOTYPIC CORRECTION OF VON WILLEBRAND DISEASE TYPE 3 BLOOD-DERIVED ENDOTHELIAL CELLS WITH LENTIVIRAL VECTORS EXPRESSING VON WILLEBRAND FACTOR

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Von Willebrand disease (VWD) is an inherited bleeding disorder, caused by quantitative (type 1 and 3) or qualitative (type 2) defects in von Willebrand factor (VWF). Gene therapy is an appealing strategy for treatment of VWD since it is caused by a single gene defect and since VWF is secreted into the circulation, obviating the need for targeting specific organs or tissues. However, development of gene therapy for VWD has been hampered by the considerable length of the *VWF cDNA* (8.4kb) and the inherent complexity of the VWF protein that requires extensive post-translational processing. In this study, a gene-based approach for VWD was developed using lentiviral transduction of blood-outgrowth endothelial cells (BOECs) to express functional VWF. A lentiviral vector encoding complete human VWF was used to transduce BOECs isolated from type 3 VWD dogs resulting in high transduction efficiencies (95.6±2.2%). Transduced VWD BOECs efficiently expressed functional vector-encoded VWF (4.6±0.4 U/24hr/10⁶ cells), with normal binding to GPIIb α and collagen and synthesis of a broad range of multimers resulting in phenotypic correction of these cells. These results indicate for the first time that gene therapy of type 3 VWD is feasible and that BOECs are attractive target cells for this purpose.

Adenoviral-mediated gene transfer of single chain Fv 425:sTRAIL fusion protein induces target cell restricted apoptosis in EGFR-positive tumor cells and a potent bystander effect.

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Introduction: Soluble TRAIL (sTRAIL), a recombinant form of tumor necrosis factor-related apoptosis-inducing ligand, has been shown to induce apoptosis in a wide variety of cancer cells *in vitro* and to suppress tumor growth *in vivo*. Unfortunately the specificity of sTRAIL for tumor cells is limited as several reports show toxicity of sTRAIL towards normal cells. Genetic linkage of sTRAIL to an antibody-fragment specific for a tumor-associated antigen increases the specificity of sTRAIL for tumor cells and simultaneously enhances the apoptosis inducing capacity of sTRAIL by cross linking the agonistic TRAIL-receptors.

Aim: We hypothesized that an adenovirus expressing a fusion protein consisting of a Epidermal Growth Factor Receptor (EGFR)-specific single chain Fv antibody fused to sTRAIL: AdscFv425:sTRAIL would induce specific apoptosis in EGFR-positive tumor cells with a potent bystander effect

Results: AdscFv425:sTRAIL infected cells showed high expression and secretion of functional trimerized scFv425:sTRAIL. Infection of renal cell carcinoma cells with AdscFv425:sTRAIL resulted in EGFR-restricted and TRAIL-mediated apoptosis induction in 80% of the tumor cells, strongly prevailing the percentage of transduced cells.

We next investigated the bystander effect in a transwell system with AdscFv425:sTRAIL infected human umbilical vein endothelial cells (HUVEC) cells and renal carcinoma bystander cells separated by a permeable membrane. In the cancer cells 60% apoptosis was observed without any sign of viral infection with concurrently no toxicity of AdscFv425:sTRAIL towards the infected HUVEC cells.

Conclusion: From these results we conclude that AdscFv425:sTRAIL is a very potent treatment for EGFR-expressing tumor cells as it induces target cell-restricted apoptosis and massive bystander killing with no detectable toxicity towards normal cells. This suggest a putative role of adenoviral delivery of targeted sTRAIL-fusion proteins for treatment of several kinds of cancers.

Intravenous administration of the conditionally replicative adenovirus Ad5- Δ 24RGD induces regression of osteosarcoma lung metastases in an animal model

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Abstract

A major problem in the treatment of osteosarcoma is the frequent occurrence of metastases in the lungs. Consequently, an effective treatment of lung metastases would be of great benefit for osteosarcoma patients. We have previously shown that the conditionally replicative adenovirus (CRAd) Ad5- Δ 24RGD was effective in killing primary osteosarcoma cells in vitro (1). Furthermore, local treatment of subcutaneous primary OS tumors with this virus resulted in growth delay (1). The purpose of the present study was to determine whether systemically administered Ad5- Δ 24RGD could suppress the growth of human osteosarcoma lung metastases. For this purpose, we used the established osteosarcoma lung metastasis model SaOs-Im7. Oncolytic activity of Ad5- Δ 24RGD on SaOs-Im7 cells was first clearly demonstrated in vitro. Furthermore, in vivo toxicity studies in nude mice revealed that repeated intravenous administration of this oncolytic virus did not cause severe weight loss or liver damage as assessed by microscopy.

SaOs-Im7 osteosarcoma lung metastases bearing mice were treated at week 1,2 and 3 (group I) or at week 5, 6 and 7 (group II) post tumor cell injection with 1×10^9 plaque forming units (pfu) Ad5- Δ 24RGD. Animals were analyzed at ten weeks post tumor cell injection. Group I treated animals did not significantly differ from PBS injected controls. In contrast, mice treated at weeks 5-7 showed a significantly reduced lung weight (decrease of tumor mass, $p < 0.05$), a significant increase of 10% total body weight (decrease of disease symptoms, $p < 0.05$) and a reduced amount of lung tumor nodules

(median 60 versus ≥ 174) compared to PBS treated control animals. These findings suggest that systemic administration of Ad5- $\Delta 24$ RGD might be a promising new treatment strategy for metastatic osteosarcoma and that treatment effect is dependent on an established tumor vasculature.

Reference:

1. Witlox AM, Van Beusechem VW, Molenaar B, et al. Conditionally Replicative Adenovirus with Tropism Expanded towards Integrins Inhibits Osteosarcoma Tumor Growth in Vitro and in Vivo. Clin Cancer Res 2004;10:61-7.

Retroviral gene transfer of T cell receptors (TCR) specific for minor histocompatibility antigens to virus-specific T cells as cellular immunotherapy of patients with relapsed hematological malignancies after allogeneic stem cell transplantation.

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Abstract

Patients with hematological malignancies can be successfully treated by T cell-depleted allogeneic stem cell transplantation (alloSCT) followed by donor lymphocyte infusion (DLI). Although this two-step procedure significantly reduces the risk and severity, Graft-versus-Host Disease (GvHD) remains an important cause of morbidity and mortality. We previously showed that functional T cells with redirected anti-leukemic reactivity can be generated by transfer of TCRs specific for minor histocompatibility antigens (mHags) to peripheral blood lymphocytes as well as CMV-specific T cells. By introducing TCRs into CMV- and EBV-specific T cells, T cells with proper memory/effector phenotypes are targeted, and due to virus persistence, these T cells may show prolonged survival in vivo. Moreover, adoptive transfer of virus-specific T cells to patients treated with alloSCT has a minimal risk for alloreactivity. The purpose of this study is to develop a method for the efficient generation of TCR-transduced virus-specific T cells for cellular immunotherapy of patients with relapsed hematological malignancies after alloSCT.

For clinical application, single retroviral vectors encoding the alpha as well as beta chains of TCRs specific for mHags are required. Various single retroviral vectors were constructed containing the alpha and beta chains of the TCR specific for mHag HA-2 either

linked by an IRES element or by a 50-bp sequence encoding a "self-cleaving" 2A-like peptide. Introduction of a 2A-like sequence allows additional linkage of a selection marker gene, such as the low affinity nerve growth factor receptor (LNGFR). After transduction of Jurkat T cells lacking endogenous TCR expression as well as virus-specific T cells, highest TCR expression levels were obtained with retroviral constructs expressing the TCR alpha and beta chains linked by an IRES element. Furthermore, a method for the efficient generation of TCR-transduced virus-specific T cell lines has been developed. From various human individuals, CD8⁺ cells were stimulated with a mixture of EBV and CMV peptides and at day 3 transduced with retroviral vectors encoding the HA-2-specific TCR. At day 8-10, the TCR-transduced T cell lines were shown to contain 20-80% virus-specific T cells with transduction efficiencies varying between 10-50%. Moreover, functional analysis demonstrated that the TCR-transduced T cell lines recognized and lysed target cells expressing viral antigens as well as endogenously-processed HA-2. These data show that TCR-transduced virus-specific T cell lines with dual specificities for viral antigens and mHags can be efficiently generated by transduction of CD8⁺ cells stimulated with viral peptides with a retroviral vector encoding the TCR alpha and beta chains linked by an IRES element.

Lentiviral vectors for the generation of tumour targeted macrophages in immune competent mice

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Chimeric immune receptors (CIR), consisting of a single chain antibody fragment directed towards a tumour antigen fused to an immune cell activating signal transduction domain, have shown promise in directing an effective and specific cellular immune response towards tumour cells. These results have been predominantly obtained in the context of (cytotoxic) T-cell mediated anti-tumour therapy. Monocytes may form an equally promising target for CIR gene transfer as these cells are known to effectively home to- and infiltrate solid tumour masses. However, once the monocytes enter the tumour and differentiate into macrophages they typically adopt the, tumour supporting, alternative (like) state of activation (also referred to as M2 or Type 2). This unwanted switch to macrophages with an M2 like phenotype may be countered by the introduction of CIR.

We have constructed a CIR consisting of the Macrophage activating receptor FcγRI (CD64) fused to MFE23, a single chain antibody fragment that specifically recognizes the Carcinoembryonic antigen (CEA). Adenoviral transfer of the MFE23.CD64 CIR into human monocytes effectively and specifically redirected immune responses to human tumour cells both in vitro and, in athymic nude mice, in vivo.

To further investigate the potential of genetically modified macrophages and other myeloid cells for the treatment of solid cancers, immune competent murine models are needed. Lentiviral vectors have the unique ability to integrate their genome into non-dividing cells making them particularly suited for the transduction of stem- and progenitor cells. Therefore we are using these vectors to establish gene modified murine immune systems in which the full effect of the introduction of CIRs in any haematopoietic lineage can be studied. Future direction for this work is to restrict transgene expression to cells of myeloid origin. To facilitate this we have constructed a lentiviral vector containing the MRP8 promoter (derived from the human myeloid related protein 8 gene) to drive marker genes and potentially chimeric immune receptors.

Title: Pseudotyping lentiviral vectors with a modified GP64 envelope proteins redirects gene transfer *in vitro* and *in vivo*.

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Body: In rodents, systemic administration of lentiviral vectors results in poor gene transfer to hepatocytes, with the nonparenchymal liver cells preferentially transduced. Lentiviral vectors often are pseudotyped with envelope proteins capable of gene transfer to a broad range of cell types. Restricted gene transfer to hepatocytes is important for improving transduction efficiency and long-term transgene expression.

The GP64 protein from the baculovirus *Autographa californica* multiple nuclear polyhedrosisvirus has been successfully used for the surface display of amino terminal fusion proteins and peptide display in baculovirus. To target gene transfer to hepatocytes two strategies were employed. A peptide fragment of the Hepatitis B Virus (HBV) PreS1 protein, shown to bind to hepatocytes, was inserted internally into GP64 to make PreS1-GP64. Secondly, the amino terminal region of the hepatocyte specific Sendai Virus fusion protein (SV-F), F2, was fused in frame to the amino terminus of GP64 to make Sendai-GP64.

PreS1-GP64 pseudotyped lentiviral displayed an increased affinity for hepatoma cells as compared to wild type GP64, with undetectable gene transfer to HeLa cells (a cervical carcinoma cell line). Viral titers with PreS1 were on the order of 40 fold lower than wild type GP64 on HepG2 cells (a hepatoma cell line). Characterization of the PreS1-GP64 protein was performed confirming both expression and trimerization.

The SV-F envelope protein has been shown to be liver specific, but does not efficiently pseudotype lentiviral vectors. The Sendai-GP64 pseudotyped lentiviral vectors showed specific gene transfer to HepG2 hepatoma cells, with no detectable transduction of the HeLa cervical carcinoma cell line. Viral titers on HepG2 cells of the Sendai-GP64 lentiviral vectors were 100 fold lower as compared to GP64. To improve viral titers, different ratios 2:1, 9:1, 29:1, and 99:1, of Sendai-GP64 to GP64 envelope plasmids were used to produce lentiviral vectors. The ratio of HepG2 to HeLa titers was compared for all viruses produced: 6.1 (GP64), 7.4 (2:1), 15.8 (9:1), 28.9 (29:1), and 30.7 (99:1). An increase in the relative amount of Sendai-GP64 envelope increased the affinity for HepG2 cells. We further observed an improvement in viral titers on HepG2 cells from $2.7 \times 10^4 \pm 1.3 \times 10^4$ HepG2 TU/ml with Sendai-GP64 to $5.1 \times 10^5 \pm 2.7 \times 10^5$ (29:1 Sendai-GP64:GP64).

Based on the improved affinity for HepG2 cells and viral titers the 29:1 Sendai-GP64:GP64 lentiviral vectors were further investigated for *in vivo* gene transfer to hepatocytes versus wild type GP64. Equivalent HepG2 titers of GP64 or 29:1 Sendai-GP64:GP64 lentiviral vectors were injected into the portal vein of FVB mice. The Sendai-GP64:GP64 (29:1) lentiviral vectors resulted in a significant increase ($p=0.005$) in the ratio of transduced hepatocytes to transduced nonparenchymal liver cells 0.13 ± 0.05 (GP64 $n = 5$) versus 0.52 ± 0.19 (Sendai-GP64/GP64 29:1 $n=7$) as determined from counting GFP expressing cells in liver sections.

This work demonstrates the potential of the GP64 envelope protein as a platform to redirect lentiviral vectors for *in vitro* and *in vivo* gene transfer.

ONCOLYTIC ADENOVIRUSES REDIRECTED WITH A TUMOR-SPECIFIC T-CELL RECEPTOR

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Abstract

Oncolytic adenoviruses constitute promising therapeutics to treat cancer patients, however their safety and specificity needs to be further improved prior to clinical application. This can be achieved via ablation of the natural tropism of adenoviruses and redirecting them against new cellular target molecules. In this respect T-cell receptors (TCR) specific for Major Histocompatibility class (MHC) I and II restricted tumor antigens are potential candidates for adenoviral retargeting. These retargeting structures may not be limited by suboptimal refolding as is observed for antibodies.

We genetically replaced the adenoviral fiber knob with a single-chain (sc)TCR molecule with specificity for the melanoma cancer testis antigen MAGE-A1, presented by HLA-A1, and an extrinsic trimerization motif in a replicating Ad5 vector (Ad5.R1-scTCR). The initial production of the recombinant virus required a novel producer cell line that expressed a membrane anchored antibody-based hexon-specific receptor (293T AdR). In fact, this new production system allowed CAR- or target antigen independent propagation of Ad5.R1-scTCR. Importantly, infection with adenovirus bearing the scTCR-based fiber resulted in an efficient eradication of target tumor cells. The infection was antigen-specific since only HLA-A1^{pos}/MAGE-A1^{pos} melanoma cells were killed. We believe that successful incorporation of TCRs with specificity for MHC-restricted cancer testis antigens, which represent a class of antigens that are uniquely expressed in tumor cells on the adenoviral fiber opens new and safer strategies for tumor-specific retargeting of oncolytic adenoviruses, providing a versatile tool for future clinical application.