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Engineered mesenchymal stem cells as vectors in a suicide gene therapy against preclinical murine models for solid tumors☆

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ABSTRACT

Gene-directed enzyme pro-drug therapy (GDEPT) consists of expressing, in tumor cells, a suicide gene which converts a pro-drug into cytotoxic metabolites, *in situ*. In a previous work, we demonstrated that the combination of the suicide gene CYP2B6TM-RED (a fusion of a triple mutant of CYP2B6 with NADPH cytochrome P450 reductase) and cyclophosphamide (CPA) constituted a powerful treatment for solid tumors. In this work, we investigated the use of mesenchymal stem cells (MSCs) as cellular vehicles for the delivery of our suicide gene. MSCs were genetically engineered *ex-vivo* to stably express CYP2B6TM-RED. *Ex vivo* and *in vivo* investigations showed that MSCs expressing CYP2B6TM-RED were able 1) to bioactivate CPA and produce local cytotoxic metabolites in tumor sites and 2) to destroy neighboring tumor cells through a bystander effect. Intratumoral injections of CYP2B6TM-RED-MSCs and CPA completely eradicated tumors in 33% of mice without recurrence after 6 months. Rechallenge experiments demonstrated an efficient immune response. These data suggest that MSCs expressing CYP2B6TM-RED with CPA could represent a promising treatment for solid tumors to test in future clinical trials.

1. Introduction

Gene-directed enzyme prodrug therapy (GDEPT) utilizes the expression of a suicide gene in tumor cells for the *in situ* conversion of a pro-drug into cytotoxic metabolites. In a previous work, we demonstrated that the combination of a modified CYP2B6, CYP2B6TM-RED, as a suicide gene, and cyclophosphamide (CPA), as a pro-drug, constituted a powerful treatment for solid tumors [1]. The efficacy of this combination mainly was due to i) an optimized suicide gene able to metabolize very efficiently CPA, ii) an efficient by-stander effect, iii) the development of an anti-tumor immune response and iv) an efficient and specific delivery of the suicide gene to the tumors. Although we clearly achieved the three first points, the last point, gene delivery, had leeway for improvement. Several methods for gene delivery have been reported in the literature [2]. Nonviral vectors have several advantages such as low immunogenicity, practically unlimited packaging capacity for genetic material, and simple and low-cost production which makes them suitable for large-scale production and potentially safe for clinical use [3]. However, these vectors possess low gene transfer efficiency as well as only transient or steadily declining gene expression. Viral vectors have both low packaging capacity and relatively high production costs which constitute limitations for their marketing [4]. Recently, therapies based on genetically engineered mesenchymal stem cells (MSCs), which express a suicide gene, have received a great deal of attention because of their therapeutic potential to treat solid tumors [5–7]. Indeed, MSCs possess the specific ability to home and nest into tumors due to the inflammatory mediators which are found at tumor sites [8–12].

MSCs can be easily isolated, from tissues such as bone marrow (BM-MSCs) and adipose tissue (ACS), expanded in culture and transduced efficiently with recombinant viral vectors which permits stable expression of the suicide gene [13,14]. Once the transduction has been performed, the most efficient clone to bioactivate the prodrug can be selected.

In this work, commercially available murine MSCs were genetically engineered *ex-vivo* to stably express CYP2B6TM-RED. The efficacy of these MSCs to bioactivate CPA and destroy neighboring tumor cells

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through a by-stander effect was investigated, *ex vivo* and *in vivo*. Re-challenge experiments were performed to determine whether the MSCs triggered an immunological response.

We found that MSCs remained in the tumor site following their injection into tumors. Complete tumor eradication was observed in 33% of mice following intratumoral injections of MSCs-CYP2B6TM-RED and CPA. There was no tumor recurrence six months later. An immunological response similar to that documented in our previous work [1] was observed in the mice cured with MSCs.

2. Materials and methods

2.1. Cell cultures

TC1 cells, derived from a murine lung epithelial-cell line which express the HPV16 E6-E7 proteins and TC1-Luc2 cells which express firefly luciferase [15] were cultured as previously described [1].

Murine mesenchymal stem cells (MSCs) from the bone-marrow of C57BL/6 mice were provided by Life technologies (GIBCO, Saint Aubin, France) and were cultured in DMEM High glucose medium with L-glutamine, without sodium pyruvate or HEPES (GIBCO), supplemented with 10% fetal calf serum, streptomycin (50 μg/mL), penicillin (200 UI/mL), fungizone (0.5 μg/mL) and sodium pyruvate (1 mM). DLD-1, a human colorectal cancer cell, and HCC 1143, a human breast cancer cell line, were cultured in RPMI 1640 medium with L-glutamine, without sodium pyruvate and HEPES (GIBCO) supplemented with 10% fetal calf serum, streptomycin (50 μg/mL), penicillin (200 UI/mL), fungizone (0.5 μg/mL). Cal-27, a human head and neck cancer line, was cultured in DMEM High glucose medium with L-glutamine, without sodium pyruvate or HEPES, supplemented with 10% fetal calf serum, streptomycin (50 μg/mL) and penicillin (200 UI/mL). All human cell lines were provided by ATCC (LGC Standards, Manassas, VA, USA).

2.2. Lentiviral production and transduction, generation of clones (F5 and 42)

pHIV-EF1L-thy1-W is an HIV-derived vector construct derived from the HIV-1 self-inactivating (SIN) vector backbone pRRL-SIN18-cPPT-hPGK-EGFP-WPRE (pHIV-hPGK-GFPW+) [16].

LV-CYP2B6TM-RED 3Ser + Th (LV-CYP2B6TM-RED) and LV-Luc2, which expressed luciferase, constructs were derived from the pHIV-EF1L-thy1/GFP-W+ (LV-Thy1) construct as previously described [1]. LV-Thy1 was used as negative control. Briefly, the thy1 gene was excised from pHIV-EF1L-thy1/GFP-W+ and the CYP2B6TM-RED sequences were then inserted between the BamH1 and MluI restriction sites. The cassettes were under the control of the full-length elongation factor 1 alpha promoter (EF1L).

MSCs were transduced twice with lentiviral particles. Human cell lines (DLD-1, Cal-27 and HCC 1143) were transduced once by lentiviral particle LV-CYP2B6TM-RED as previously described [1]. CYP2B6TM-RED expression was monitored by RT-PCR and by MTS cytotoxic assays (Promega, Charbonnieres, France) in the presence of CPA (0 to 3 mM, data not shown). Luciferase expression was monitored with the luciferase assay system (Promega) according to the manufacturer's instructions and luminescence was detected with a luminometer (EnVision Multilabel Reader, Perkin Elmer, Waltham, Massachusetts, USA).

MSCs clones infected with LV-Luc2 or LV-CYP2B6TM-RED were selected with two series of limiting dilutions: cells were seeded at 0.5 cells per well in 96-well plates. Among the clones obtained, one clone per construct (clone F5 for MSCs-Luc2 and clone 42 for MSCs-CYP2B6TM-RED) was chosen on the basis of the highest level of transgene catalytic expression as determined by: 1) luciferase expression or 2) MTS cytotoxic tests in the presence of CPA (Promega).

2.3. Evaluation of in vitro by-stander effects

MSCs-CYP2B6TM-RED (pool or clone 42) and TC1-Luc2 or human cell lines (DLD-1, Cal-27 and HCC 1143) were seeded in triplicate into 96-well plates at different ratios, in MSCs culture medium. 24 h later, cells were exposed for 3 days to CPA (0 to 3 mM) and cell viability was measured by MTS assay (Promega).

2.4. Cytotoxic assays in human cell lines

Transduced and non-transduced DLD-1, Cal-27 and HCC 1143 cells were seeded into 96-well plates (5×10^3 cell per well). 24 h later, cells were exposed for 3 days to CPA (0 to 3 mM) and cell viability was measured by MTS assays.

2.5. Animal models

In vivo studies were performed on 10-week-old female C57BL/6 mice (Janvier, Saint-Berthevin, France). All animal experiments were conducted in accordance with ethical guidelines and were approved by the animal ethics committee of the Paris Descartes University (CEEA34.IW.031.11, CEEA34.IDW.097.12).

2.6. Effects of different doses of CPA and re-challenge

5×10^5 TC1-Luc2 or TC1-Luc2-CYP2B6TM-RED cells were diluted into 50 μL of sterile phosphate buffered saline (PBS) and injected into the subcutaneous tissue of the left flanks of mice as previously described [1]. When the tumor size approached 500 mm^3, mice bearing either TC1-Luc2 or TC1-Luc2-CYP2B6TM-RED tumors were divided into four groups each and given intra-peritoneal injections of 0 mg/Kg, 45 mg/Kg, 90 mg/Kg or 140 mg/Kg of CPA (Endoxan, Baxter, Maurepas, France) diluted in sterile PBS. CPA was administered once a week for 4 weeks. Tumor growth was evaluated by Vernier caliper measurement and was calculated according to the formula: volume (mm^3) = length × $width^2$ / 2.

2.6.1. Re-challenge

Mice that survived treatment with 90 mg/Kg (n = 6) or 140 mg/Kg CPA (n = 8) were divided in two groups. 24 h before both groups of mice received a subcutaneous injection, into the right flank, of 2.5×10^5 TC1-Luc2 cells diluted in 50 μL PBS, the mice of one of the groups were injected intra-peritoneally with anti-CD8 antibodies (100 μg/mouse, Euromedex, Strasbourg, France). To maintain depletion of CD8, anti-CD8 antibody injections were repeated once a week until the sacrifice of the mice. Control mice were the same age as the surviving mice with no history of tumor cell injection or CPA treatment and they were injected subcutaneously with 2.5×10^5 TC1-Luc2 cells diluted in 50 μL PBS without any other treatment. Tumor growth was evaluated by luminescence imaging. Twenty minutes after intra-peritoneal injection of 200 μL of 33 μM Luciferin (Synchem, Chicago, IL, USA) and under isoflurane anesthesia, luminescence was detected using a Biospace Photon Imager camera (BiospaceLab, Paris, France). The photos were analyzed using image-analysis software (BiospaceLab).

2.7. Lentiviral injection transduction efficacy in vivo

Given that Thy1 is expressed on the cell surface and is monitored readily by FACS, LV-Thy1 was used to assess the efficacy of lentiviral transduction in the tumors. In mice bearing TC1-Luc2 tumors (500 $mm^{3)}$, 50 μL of PBS or LV-Thy1 (3×10^7 lentiviral particles) were injected into tumors 3 times at intervals of two day. Animals were euthanized 4 days after last injection of lentiviral particles when the tumor size reached 1000 mm^3.

Tumors were dissociated using 1 mg/mL of collagenase IV (Sigma-Aldrich) and 0.2 mg/mL of DNase (Roche). After counting, the cells were stained using Fixable Viability Dye eFluor780 at 2–8 °C for