Mesenchymal stromal cells producing TNFα lack inhibitory effect against A375 experimental lung metastases

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Cell-based anticancer therapy using mesenchymal stromal cells (MSCs) engineered to express therapeutic genes has a potential to target the cancer cells *in vivo*. Metastatic dissemination of melanoma remains a serious problem in the treatment. In our previous work we used MSCs overexpressing gene for tumor necrosis factor α (TNFα; MSCs/TNFα), and we achieved inhibition of melanoma xenograft growth when engineered MCSs/TNFα were coinjected with tumor cells subcutaneously. The TNFα as a pleiotropic cytokine induces apoptosis of tumor cells, creates "tumor resistant" microenvironment, enhances immune response and can have tumor destructive capacity in selected tumor types, especially in tumors of mesodermal origin.

In this study we investigated the possibility of intravenously administered MCSs/TNFα to inhibit metastatic spread of A375 melanoma cells in the lungs. We confirmed elevated expression of TNFα transgene in the lung tissue 20 days after MCSs/TNFα intravenous infusion. We also documented that constitutive expression of TNFα transgene is able to neutralize the supportive effect of MSCs on melanoma cells growth. Metastatic spread of A375 melanoma cells in the lung was inhibited approximately to 50% after MCSs/TNFα *i.v.* administration in comparison to control group with parental MSCs supporting tumor growth. In conclusion, engineered MCSs/TNFα administered intravenously did not demonstrate significant antitumor effect against experimental melanoma lung metastases in this model settings.

Key words: mesenchymal stem cell, tumor necrosis factor alpha, cancer gene therapy, melanoma, experimental metastases

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine with multiple activities, both antitumorigenic and protumorigenic [1] and as a central proinflamatory cytokine, it modulates tumor microenvironment. The TNFα has tumor destructive capacity [2] and creates microenvironment in which cancer cells are unable to form tumors [3], on the other hand, it contributes to a tumor permissive microenvironment and tumor progression [4]. In the clinic, the TNFα protein in combination with melphalan was used in local administration in isolated limb perfusion of melanoma and soft tissue sarcoma patients. This treatment modality resulted in a local control of the disease [5, 6]. Presence of TNFα enhanced antitumor immune response and activation of tumor-infiltrating CD8+ T lymphocytes [7]. The TNFα exposure strongly sensitized tumor cells to cisplatin-mediated killing causing synergistic cell death. The TNFα could sensitize tumor cells also for topotecan- and carboplatin-induced tumor cell death but not for paclitaxel-induced apoptosis [8].

Metastatic melanoma often spreads to lungs and liver and represents one of the most aggressive and difficult-to-treat disease. The tumor-homing ability of mesenchymal stromal cells (MSCs) allows targeted delivery and production of biological agents locally at the site of tumor growth including disseminated metastatic foci [9]. Engineered MSCs expressing suicide prodrug-converting genes were successfully used in experimental cancer therapy of xenografts of various origin and lung metastases when administered intravenously or locally [10-14]. Other groups proved that MSCs engineered to express factors, such as interferons, can be used for targeted delivery to experimental lung metastases derived from melanoma [15, 16] or prostate cancer [17]. Systemic delivery of engineered MSCs producing TNF-related apoptosis inducing ligand (TRAIL) reduced breast cancer lung metastases [18]. The use of MSCs engineered to produce combination of TRAIL plus suicide Herpes simplex virus thymidine kinase (HSV-TK) resulted in a long-term

remission of experimental lung metastases of renal cell carcinoma [19].

As reported by Lee *et al.*, MSCs pre-activated with TNFα were induced to secrete tumor suppressive proteins TRAIL and DKK3. Then, TNFα- pre-activated MSCs administered intravenously in combination with sub-toxic dose of doxorubicin inhibited progression of lung metastases derived from breast carcinoma cells and overcame TRAIL resistance of the tumor cells [20, 21]. Our previous work revealed that adipose-tissue derived MSCs (AT-MSCs) tolerated and constitutively expressed human gene for TNFα (hTNFα; AT-MSCs/hTNFα), and significantly suppressed the growth of subcutaneous melanoma xenografts when coinjected locally with tumor cells [22]. Additionally, tumor cells stably overexpressing TNFα fail to form tumors in xenografted mice, conferring 100% protection against tumorigenesis [3].

In the presented study, we tested whether engineered AT-MSCs secreting hTNFα could demonstrate tumor homing ability, lose supportive effect on melanoma growth and show antitumor effect when administered intravenously in aggressive A375 melanoma lung metastatic model. With TNFα cell-based experimental gene therapy we intended to avoid the systemic toxicity of TNFα and selectively increase TNFα concentration at the site of tumor.

Material and methods

Cell cultures and chemicals. Human melanoma cells EGFP-A375iv [13] derived from A375 cell line (ECACC 88113005) were maintained in high-glucose (4.5mg/ml) Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 5% fetal bovine serum (FBS) (Biochrom AG), antibiotic/antimycotic mix (10 000 IU/ml penicillin; 5μg/ml streptomycin; 2.5μg/ml amphotericin) and 2mM glutamine. The AT-MSCs were isolated and expanded as described previously [10] from healthy individuals undergoing cosmetic liposuction or plastic surgery. For the cultivation of the AT-MSCs, low-glucose (1.0 mg/ml) DMEM supplemented with 5% FBS, 5% HyClone AdvanceSTEM Mesenchymal Stem Cell Growth Supplement (Thermo Fisher Scientific, Waltham, MA, USA) and antibiotic/antimycotic mix was used. The cells were maintained in humidified atmosphere at 37 °C and 5% CO_2. All chemicals were purchased from Sigma (St Louis, MO, USA) if not stated otherwise.

Retrovirus production and retroviral transduction. Subconfluent cultures of proliferating AT-MSCs were transduced with Moloney Murine Leukaemia Virus- derived replication deficient retroviral particles ST40hTNFα bearing human TNFα transgene as described previously [22].

Quantitative real-time PCR (qPCR) detection of experimental lung metastases and hTNFα transgene expression. Quantitative real-time duplex PCR analysis (qPCR) was used for determination of EGFP-A375iv experimental metastases spread in mouse lungs according [14, 23]. Total gDNA was isolated from lung tissue (50 ng) by NucleoSpin® Tissue kit

(Machery-Nagel, Dueren, Germany) and 100 ng of gDNA was used per PCR reaction. TaqMan fluorogenic probes and primers for human specific β-globin gene and mouse specific RAPSYN gene were mixed with Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed in triplicates in Bio-Rad 96FX cycler. The analysis was done using a Bio-Rad CFX Manager software version 1.6 as normalized fold expression of the β-globin gene to the RAPSYN gene as a reference gene. Ten-fold dilutions of control gDNA of the human tumor cells EGFP-A375iv were used as standards for determination of relative content of human cells in the lung tissue. Primer and probe sequences were used as published in [14]. For detection of hTNFα expressed by engineered AT-MSCs/hTNFα in mouse lung tissues, cDNA was cleaned-up using SureClean Plus solution (Bioline, London, UK) and quantified using a NanoDrop spectrophotometer. Quantitative RT-PCR was performed using Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). Per one PCR reaction, 40 ng of purified cDNA was used to evaluate the differences in the hTNFα expression between individual lung samples. GAPDH gene was used as a reference gene. Primer sequences for hTNFα and GAPDH gene were used as published in [22]. All oligonucleotides were synthesized by Metabion, Int. (Martinsried, Germany).

Flow cytometry detection of experimental lung metastases. Lung tissue was mechanically dissociated by gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), and digested by 1:1 trypsin-accutase mixture for 40 minutes at 37°C. Single cell suspension was prepared using 30 μm filters and stained with DAPI as published in [14]. Samples were analysed using the BD FACSCanto II analyser (Beckton Dickinson, Franklin Lakes, NJ, USA) equipped with BD FACSDiva software.

***In vivo* animal model/lung colonization assay.** Six to eight weeks old SCID/bg mice were used in accordance with the institutional guidelines under the approved protocols. The melanoma EGFP-A375iv cells (0,75x10⁶) resuspended in 100 μl PBS were administered intravenously via lateral tail vein to induce lungs' xenografts. Mice were randomly divided into 4 groups (n = 4-7) according to the treatment. Engineered AT-MSCs/hTNFα or parental AT-MSCs were administered intravenously (1×10⁶ cells in 200 μl PBS) once – on day 7 (single dose group) or twice – on days 7 and 14 (double dose group) after the injection of the EGFP-A375iv tumor cells. Mice were sacrificed on day 26-28. Lungs were examined for macroscopically visible tumor foci formation and the samples were taken for the subsequent molecular-biological and flow cytometric analysis. Enlarged lymph nodes were analysed for presence of human sequences too.

Statistical analysis. The Student's two sample *t*-test was used for testing the difference in means of two samples and the Mann-Whitney *U* test was used to perform a two-sided test to compare two independent groups. The *p*- values < 0.05 were considered statistically significant.
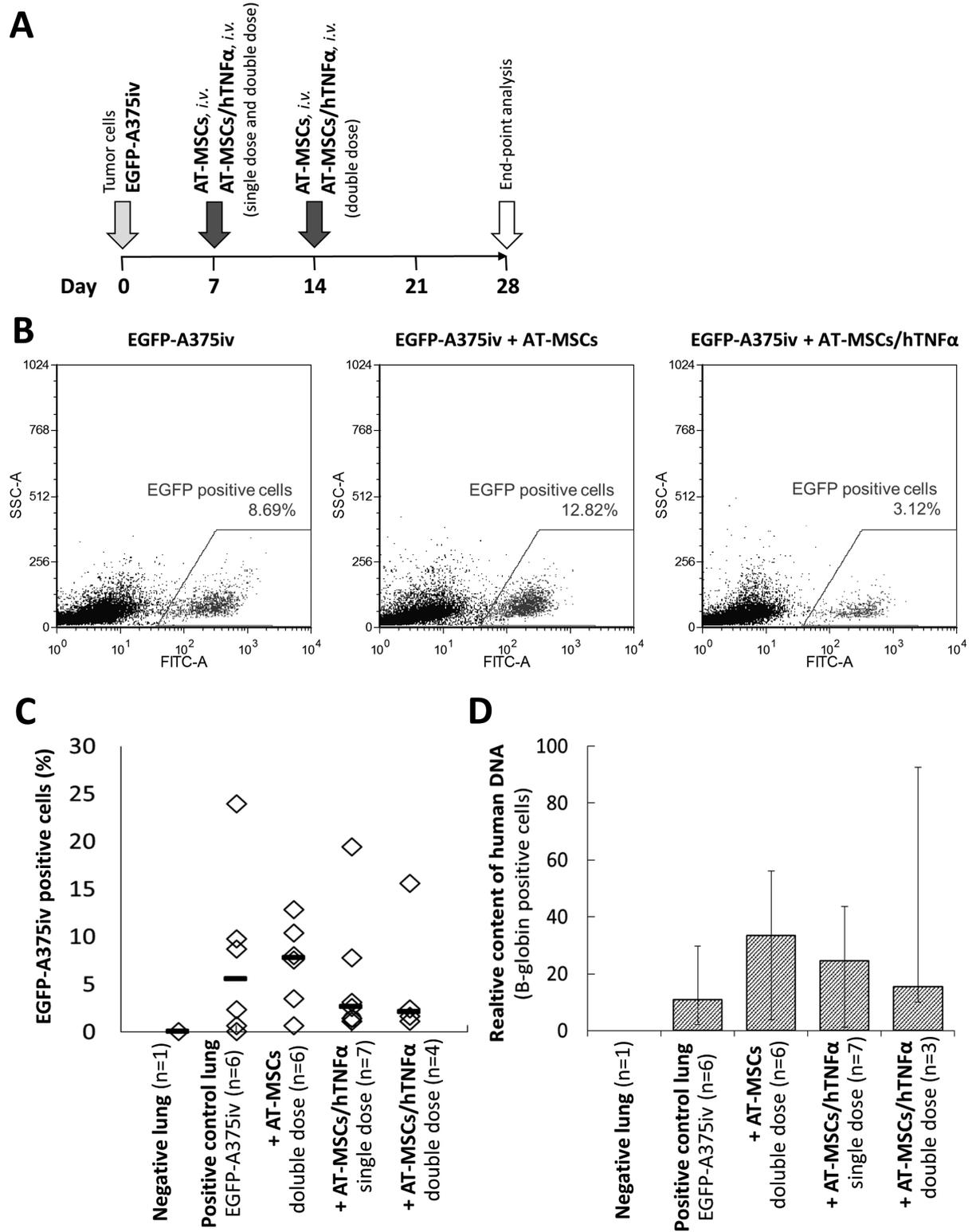
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Figure 1. Experimental lung metastases derived from melanoma EFGP-A375iv cells after intravenous administration of engineered AT-MSCs/hTNFα. **(A)** Experimental design scheme. **(B)** Representative flow cytometry dotplots of single cell suspension prepared from mouse lungs, EGFP positive cells originate from EGFP-A375iv derived lung metastases. **(C)** EGFP-positive A375iv melanoma cells detected in individual mouse lung samples by flow cytometry; bars represent medians **(D)** Molecular-biological analysis of the lungs; human cells EGFP-A375iv and AT-MSCs engrafted in the lung detected by qPCR for human specific β-globin gene in the lung tissue genomic DNA (gDNA). The proportion of human cells was calculated according to the calibration curve generated from serial dilutions of standard human gDNA. Medians ± mininal and maximal values.

Results

The A375 melanoma cell line variant EGFP-A375iv is characterized by more aggressive metastatic potential and massive tumor infiltration to the lungs and lymph nodes [13]. The EGFP-A375iv cells were injected intravenously into immunodeficient SCID mice (on day 0) and formed extensive tumor foci in the lungs within 30 days. In order to evaluate the possible effect of engineered AT-MSCs/hTNFα, MSCs were administered *i.v.* as single dose on day 7 and as double dose on days 7 and 14 (Fig. 1A). Secretion of human TNFα protein by engineered AT-MSCs/hTNFα was confirmed by ELISA method with level of secreted TNFα reaching 15 ng/ml as reported in our previous study [22]. Animals tolerated the *i.v.* injections of the 1×10^6 AT-MSCs secreting human TNFα well, similar to the parental AT-MSCs injections. At the endpoint on day 26-28, all animals of the four tested groups developed lung metastases to an approximately same degree when examined macroscopically. The flow cytometry analysis of the lung tissue for the presence of EGFP-positive A375iv melanoma cells (Fig. 1B, 1C) c confirmed infiltration of the tumor cells. In the control group with EGFP-A375iv cells, the median value of infiltration was 5.53%. Higher median infiltration of 7.77% was detected in the group with EGFP-A375iv plus AT-MSCs parental cells, indicating supportive effect of AT-MSCs on melanoma cell growth. For the last two tested groups with single and double dose of engineered AT-MSCs/hTNFα, infiltration of EGFP-positive cells was lower in comparison with control group with only EGFP-A375iv tumor cells injected and the difference was statistically non-significant. The 2.58% infiltration for single dose and 2.05% for double dose was detected.

Quantitative PCR for human β-globin gene using tissue genomic DNA as a template was used to determine the level of human cell infiltration of the lungs and/or lymph nodes (Fig. 1D). In comparison to flow cytometry method, RT-qPCR was more sensitive and detected higher percentage of human cells, both EGFP-A375iv and AT-MSCs. The highest proportion of human cells in the lungs was in the group with *i.v.* administered parental AT-MSCs indicating supportive effect of AT-MSCs on melanoma cell growth. Increased tumor infiltration was documented also in the groups with single dose and double dose of engineered AT-MSCs/hTNFα in comparison to control group with no AT-MSCs injected (Fig. 1D).

In the control groups with no AT-MSCs and with parental AT-MSCs administered, lymphatic node infiltration was detected in 4 out of 12 animals. These lymph nodes were found to be positive for human gDNA. In the groups with *i.v.* administered AT-MSCs/hTNFα, one out of 11 animals developed hypertrophic lymph node but this tissue was negative for human gDNA.

In order to monitor the residual expression of hTNFα transgene in the lung after *i.v.* administration of AT-MSCs/hTNFα at the endpoint of the experiment, RT-qPCR method was used (Fig. 2). In 5 out of 11 tested lung tissues, hTNFα expression
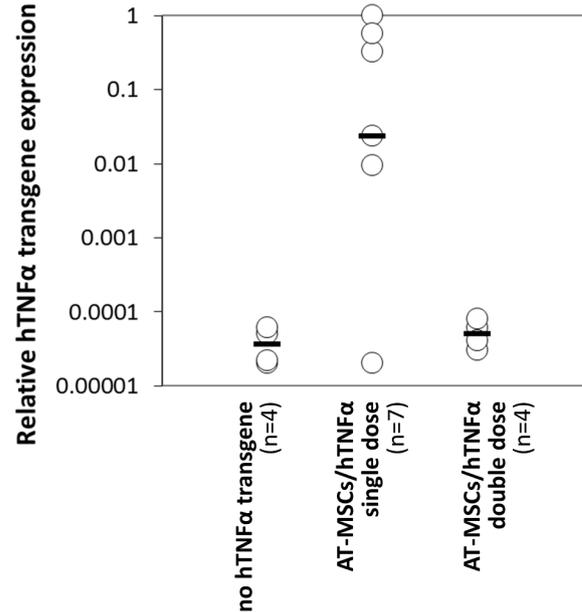

Figure 2. Expression of hTNFα transgene detected in lung tissue after intravenous administration of engineered AT-MSCs/hTNFα. RT-qPCR analysis of lung tissue at the endpoint on day 26-28.

was significantly elevated up to one thousand times in comparison to background expression level detected in control groups with no or parental AT-MSCs. Significantly increased expression of hTNFα transgene indicates its specific expression from provirus and also indicates that AT-MSCs homed and engrafted in the lung. Number of *i.v.* administrations did not influence the hTNFα expression in this experiment. Human specific GAPDH housekeeping gene was confirmed among all tested samples, indicating the presence of human cells EGFP-A375iv, AT-MSCs or AT-MSCs/hTNFα and no GAPDH expression was found in control SCID mouse lungs with no human cells injected.

Discussion

Genetically engineered MSCs have considerable therapeutic potential in a wide range of human diseases including cancer. In the current study we have used mouse model of experimental A375 melanoma lung metastases to investigate the antimetastatic effect of AT-MSCs constitutively expressing hTNFα protein as a potential therapeutic gene after intravenous administration. We have previously reported that AT-MSCs/hTNFα significantly inhibited xenograft tumor growth when coinjected together with melanoma cells subcutaneously [22].

Our data demonstrated that AT-MSCs/hTNFα administered *i.v.* had limited effect on experimental lung metastases derived from aggressive variant of melanoma A375 cells. They could not suppress the lung colonisation and tumor foci formation as in the case when AT-MSCs are engineered to express suicide

genes [13, 14]. No significant difference among the groups (parental AT-MSCs vs. engineered AT-MSCs/hTNFα) was found after the flow cytometry and molecular-biological qPCR analysis. Diverse sensitivity of this two methods was observed during experiments, but proportional differences among the groups remained the same the same (Fig. 1C, 1D). The supportive effect of AT-MSCs on the growth of A375 melanoma lung metastases was observed using both analyses. Also the neutralisation of the supportive effect of AT-MSCs constitutively secreting hTNFα on the melanoma tumor growth [24] was noticed, as it was documented in our previous work [22]. In engineered AT-MSCs/hTNFα, the hTNFα transgene is expressed from provirus, from strong retroviral LTR promoter of Moloney Murine Leukaemia Virus. The expression is stable and long-term without affecting viability of transduced AT-MSCs. The expression of hTNFα was detectable 20 days post *i.v.* administration in mouse lungs using RT-qPCR method and it could be increased up to more than thousand times in comparison to control group, but it seems to be not enough to reduce the growth of aggressive lung metastases. Evidenced hTNFα transgene expression after 20 days indicates that AT-MSCs were tracked in the lungs and home to the site of lung metastases, similarly as documented in other works [14, 25].

In presented study we have used SCID/bg mice, an immunodeficient model organism. We suppose that the full therapeutic potential of AT-MSCs/hTNFα could not be manifested in this experiment as in an immunocompetent organism, because the presence of TNFα protein can aid to an advantageous activation of tumor infiltrating lymphocytes (TILs). TILs as a heterogenous population includes CD8+ cytotoxic T lymphocytes directly capable of killing tumor cells and CD4+ T helper lymphocytes secreting cytokines, which are known to be effective in melanoma treatment and can improve the immune response through immunotherapy [7, 26]. In the recent study of Dondossola *et al.*, paradoxically *i.v.* administered murine tumor cells genetically engineered to express murine TNFα were used as cellular vehicles in experimental cancer treatment. These engineered tumor cells reduced tumors, both subcutaneous and lung metastatic colonies in immunocompetent mice, but no statistically significant increase of CD4+ and CD8+ T cells was detected [27]. Monotherapy with our AT-MSCs/hTNFα probably could be more efficient and improved in combination with immune stimulants or chemotherapeutic agents (melphalan, vindesine) which have known synergic effect with TNFα in melanoma or soft tissue sarcoma treatment [28, 29]. The aim of this cell-based therapy is to circumvent severe side effects of TNFα after systemic administration and to contribute to protective anti-tumor immunosurveillance. We suppose that locally administered AT-MSCs/hTNFα can have stronger therapeutic effect on selected tumor types of mesenchymal origin, melanoma and sarcoma, in which a good response can be expected. Because of pleiotropic activity of the TNFα protein, the use of engineered mesenchymal stem cells secreting hTNFα in the future cancer therapy must be carefully considered and well-adjusted to bring benefit of this cell-based gene therapy.

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