Cytotherapy, 2018; ■■: ■■–■■

International Society ISCT Cell & Gene Therapy

Dual Effect of Glucocorticoid-Induced Tumor Necrosis Factor–Related Receptor Ligand Carrying Mesenchymal Stromal Cells on Small Cell Lung Cancer: A Preliminary *in vitro* Study

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Abstract

Background aims. TNFR family member glucocorticoid-induced tumor necrosis factor–related receptor (GITR/TNFRSF18) activation by its ligand glucocorticoid-induced TNF-related receptor ligand (GITRL) have important roles in proliferation, death and differentiation of cells. Some types of small cell lung cancers (SCLCs) express *GITR*. Because mesenchymal stromal cells (MSCs) may target tumor cells, we aimed to investigate the effect of MSCs carrying *GITRL* overexpressing plasmid on the proliferation and viability of a GITR$^+$ SCLC cell line (SCLC-21H) compared with a GITR$^-$ SCLC cell line (NCI-H82). *Methods.* Electroporation was used to transfer pGITRL (*GITRL* gene carrying plasmid) or pCR3 (mock plasmid) into MSCs. Flow cytometry and semi-quantitative polymerase chain reaction were used to characterize the transfected MSCs. Following SCLC-21H or NCI-H82 cell lines were co-cultured with pGITRL-MSCs. *Results.* Proliferation of NCI-H82 was increased in all types of co-cultures while SCLC-21H cells did not. *GITRL* expressing MSCs were able to induce cell death of SCLC-21H through the upregulation of *SIVA1* apoptosis inducing factor. *Conclusions.* The influence of MSCs on SCLC cells can vary according to the cancer cell subtypes as obtained in SCLC-21H and NCI-H82 and enabling GITR-GITRL interaction can induce cell death of SCLC cell lines.

Key Words: *apoptosis, cell-based gene delivery, genetically modified MSCs, GITRL, mesenchymal stromal cell, small cell lung cancer, SIVA*

Introduction

Lung cancer is the leading causes of cancer death worldwide [1]. About 20% of all lung cancer cases are small cell lung cancer (SCLC) [2]. The success of the SCLC therapy remains limited because of its highly aggressive nature and its resistance to apoptosis and early dissemination with survival advantage [3,4]. The major drawback is an efficient and targeted delivery of the gene of interest into malignant tissue [5]. Alternatively, genes that can promote anti-tumor immune responses have been introduced to the tumor microenvironment. Spontaneously locating in the inflammatory and/or hypoxic sites, mesenchymal stromal cells (MSCs) can be used as cellular vehicles for intra-tumoral delivery of recombinant constructs [6–11]. Lung represents a preferential compartment for MSC accumulation [12].

Upon interacting with its cognate receptor GITR/TNFRSF18 (glucocorticoid induced tumor necrosis factor receptor), GITRL can enhance immune responses [13–16]. *GITR* is widely expressed by lymphoid cells, neutrophils and macrophages, whereas its ligand is primarily found on antigen-presenting cells [17–19]. GITR-GITRL interaction promotes T-cell proliferation, enhances effector T-cell activity and suppresses regulatory T cells. GITR does not have a death domain, but its cytoplasmic domain interacts with the pro-apoptotic protein SIVA [20,21]. Stimulation of this receptor with agonistic antibodies or recombinant GITRL has been used as a therapeutic approach in various cancers including leukemia, glioma, melanoma,

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(Received 6 November 2017; accepted 2 May 2018)

ISSN 1465-3249
https://doi.org/10.1016/j.jcyt.2018.05.002

sarcoma and colorectal carcinoma [22–25]. A recent study showed that the anti-human GITR clinical candidate antibody is promising in a humanized mouse colon carcinoma model for the inhibition of tumor growth by decreasing Tregs and increasing the number of $CD8^+$ T-cell populations [26]. Most therapeutic approaches involve lymphocytic intermediates. Such an approach has also been developed by engineering dendritic cells to express *GITRL* to boost immunity in murine Lewis lung carcinoma [27]. However, moderate expression of GITR has been reported in lineages other than T cells, and the GITR/GITRL axis may have direct effects on cancer cells that express *GITR* [27]. Heterogeneity in *GITR* expression is also observed in SCLC [28].

In this study, our initial aim was to introduce the *GITRL* gene into MSCs and investigate whether this recombinant gene expression might modulate immune responses in SCLC. However, in our preliminary *in vitro* assays, this pGITRL electroporated cells displayed differential biology, and their proliferation and viability were hampered. Therefore, here we report that transient expression of *GITRL* can have direct effects in co-cultures of MSCs and SCLCs.

Methods

Cell culture and immunophenotyping

Human bone marrow–derived MSCs (BM-MSCs; Poietics) were cultured in a 3:2 mixture of Dulbecco's modified Eagle's medium (DMEM) low glucose and MCDB201 (Sigma) containing 1% L-glutamine, 1% penicillin/streptomycin (Biochrom) and 10% fetal bovine serum. The human small cell lung cancer cell lines, SCLC-21H and NCI-H82 (DSMZ) were cultured in DMEM high glucose and RPMI1640 media, respectively, supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. The cultures were established in a humidified incubator with 5% CO_2 at 37°C.

The MSCs or cancer cells were labeled with monoclonal antibodies against anti-human CD44-FITC (clone L178), CD45-FITC (clone 2D1), CD29-PE (clone MAR4), CD14-APC (clone M5E2), CD105-PE (clone 266), CD73-PE (clone AD2), CD90-PE (clone 5E10) and HLA-DR-APC (clone L243) (Becton Dickinson); GITRL-PE and GITR-APC (R & D Systems). MSCs were incubated with these antibodies or isotype controls at 4°C for 30 min. The analyses were performed on a FACSAria flow cytometry (Becton, Dickinson and Company). All the studies were conducted in matching MSC passage. Isotype controls (FITC mouse IgG1κ, PE mouse IgG1κ, APC mouse IgG1κ (Becton, Dickinson and Company) and unstained samples were used as controls. Activated T cells were used as a positive control [29].

Electroporation

MSCs were transfected by pCR3::hGITRL [30], a plasmid expressing hGITRL under the cytomegalovirus (CMV) promotor. pCR3::hGITRL carries neomycin resistance gene under SV40 promoter, which enables selection of stable transfectants. Plasmids were purified using the Endofree Plasmid Maxi kit (Qiagen). Electroporation was performed with Neon transfection system (Invitrogen) and transfection optimization to MSCs was performed by using GFP gene carrying plasmid (pMAX). Accordingly, optimal transfection conditions for MSCs (2.5 µg plasmid/2×10^5 cells, at the conditions of 1500 V, 20 ms 1 pulse) was used for the study. After electroporation, cells were cultured in antibiotic-free medium for 24 h. Selection for stable transfectants was performed by gradual increment of geneticin (G418; Sigma) concentrations (100 µg/mL to 1000 µg/mL) for 20 days. pCR3 transfected cells were used as control. Transfected MSCs were at passage 3. MSCs used in co-culture studies were at passage 4.

Characterization

Flow cytometry was used for MSC immunophenotyping as described earlier. Osteogenic and adipogenic differentiation capacity was assessed by culturing of MSCs for 21 days in the osteogenic (10 mmol/L β-glycerophosphate, 0.1 µmol/L dexamethasone and 50 µmol/L ascorbic acid) or adipogenic medium (0.5 mmol/L isobuthyl-1-methylxanthine, 0.1 µmol/L dexamethasone, 10 µg/mL insulin, and 0.2 µmol/L indomethacin) which was followed by staining with alizarin red S for osteogenic or oil red O for adipogenic differentiation. The starting cell density was 30 000 cells/well (in a six-well plate) for the differentiation experiments as two replicates with the controls. Ultrastructure analyses were performed with transmission electron microscopy (JEM 1400, Jeol). Following fixation in 2.5% glutaraldehyde in Sorenson's buffer, cells were treated with 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions and cleared in propylene oxide. Ultrathin Epon sections were stained with uranyl acetate and lead citrate. After growing the transfected MSCs in 24-well plates at 100% confluency, cells were scratched with a pipette tip for a wound-healing assay. The closure of the scratched area was checked at 0, 3, 7 and 24 h. When the scratched area was not closed the time point was extended to 52 h. Motility index was calculated by getting the average of five points ($M = 1 - Wt / W0$) [31].

Co-culture assays

Co-cultures were performed in 24-well plates at a ratio of 1:2 (4×10^4 MSCs:8×10^4 cancer cells); 4×10^4 cells