Complete regression of metastatic renal cell carcinoma by multiple injections of engineered mesenchymal stem cells expressing dodecameric TRAIL and HSV-TK

Sae Won Kim1,3, Su Jin Kim1,3, Sang Hoon Park1, Hyun Gul Yang1, Moon Cheol Kang1, Young Woo Choi1, Seong Muk Kim2, Sin-Soo Jeun2*, Young Chul Sung1*

1Division of Molecular and Life Science, Integrative Bioscience & Biotechnology, Pohang University of Science and Technology (POSTECH), Pohang, Gyeongbuk, Republic of Korea.
2Department of Neurosurgery, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, Republic of Korea.
3These authors equally contributed to this work.

Running title

Complete cure of metastatic tumor by engineered MSCs

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*Corresponding Author:

Dr. Young Chul Sung

Division of Molecular and Life Sciences, Integrative Bioscience & Biotechnology, Pohang
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**Translational relevance**

A major hurdle in the treatment of metastatic renal cell carcinoma (RCC) is the lack of therapeutic agents that can induce durable complete remission without causing systemic toxicities. In this study, we achieved complete elimination of established metastatic RCC in 100% of mice by multiple injections of xenogeneic mesenchymal stem cells (MSCs) coexpressing dodecameric TNF-related apoptosis-inducing ligand (dTRAIL) and herpes simplex virus thymidine kinase (HSV-TK) followed by ganciclovir (GCV) administrations. The utilization of MSCs as a delivery vehicle allowed site-specific secretion of therapeutic molecules into tumor sites, thereby minimizing any side effects. Moreover, the combination of dTRAIL and HSV-TK/GCV caused increased apoptosis of tumor cells as well as MSCs, resolving safety concerns about malignant transformation of therapeutic stem cells. Our findings propose a safe and effective strategy to achieve long-term remissions of metastatic RCC by simple, repeated injections of MSCs coexpressing dTRAIL and HSV-TK.
Abstract

Purpose: Durable complete remission of metastatic renal cell carcinoma (RCC) has rarely been achieved with current treatment modalities. To solve this problem, alternative therapeutic options with high efficacy and minimal side effects are strongly needed.

Experimental Design: Mesenchymal stem cells (MSCs) were engineered to coexpress dodecameric TNF-related apoptosis-inducing ligand and herpes simplex virus thymidine kinase (MSC/dTRAIL-TK). The antitumor effects of MSCs expressing dTRAIL (MSC/dTRAIL) or HSV-TK alone (MSC/TK) and MSC/dTRAIL-TK were compared on murine RCC cells using in vitro co-culture system and in vivo experimental lung metastasis model. The effects of different doses and schedules of engineered MSCs on mice survival were also evaluated.

Results: MSC/dTRAIL-TK exerted stronger apoptotic response in Renca cells than did MSC/TK or MSC/dTRAIL after ganciclovir (GCV) treatment. In vivo imaging results suggest that MSCs reside longer in the lung of metastatic tumor-bearing mice, compared to that of control mice, regardless of genetic engineering. In addition, MSC/dTRAIL-TK treatment followed by GCV administrations significantly decreased the number of tumor nodules in the lung, to a greater degree than MSC/dTRAIL or MSC/TK, and led to a prolonged survival. More importantly, the anti-metastatic effect of MSC/dTRAIL-TK was markedly enhanced by repeated injections, but not by increased dose, and resulted in 100% survival of tumor-bearing mice after three injections.

Conclusion: Sequential combination gene therapy using MSC/dTRAIL-TK achieved long-term remission of metastatic RCC without noticeable toxicity. Our findings provide an
innovative therapeutic approach to completely eradicate metastatic tumors by simple, repeated administrations of MSC/dTRAIL-TK.
Introduction

Renal cell carcinoma (RCC) is the eighth most common type of cancer and the sixth leading cause of cancer death (1, 2). Approximately 30% of patients with RCC present with metastatic disease and the 5 year survival rate of these patients is less than 10% (3, 4). Despite the recent advances in understanding genetic and epigenetic events involved in the metastasis of RCC, current therapies have shown limited success in improving the overall survival of metastatic RCC patients. As RCC generally possesses inherent resistance to chemotherapy and radiotherapy (5), other treatment modalities, such as cytokine-based immunotherapy or targeted therapy using multikinase inhibitors, have been actively investigated in the clinic. Immunotherapies using interleukin-2 (IL-2) or interferon-α have shown beneficial effects in some clinical settings, and even complete remissions in small cohort of patients using high-dose IL-2, but low response rates and systemic toxicities have limited their clinical utility (6). Tyrosine kinase inhibitors, including sunitinib and sorafenib, have substantially improved the overall survival of RCC patients, but complete remission has been rarely achieved (7). Therefore, there remains a need to develop potent therapeutic agents for metastatic RCC that can induce high response rates with minimal side effects, and most importantly, durable complete responses.

Mesenchymal stem cells (MSCs) have recently emerged as potential therapeutic agents and cellular vehicles for gene/drug delivery, and their therapeutic efficacy is currently being assessed in various disease models including inflammatory and autoimmune diseases and cancer. Two unique properties of MSCs provide them advantages over other cellular delivery vehicles, especially in the metastatic setting: (i) tumor tropic capabilities of MSCs enable target-specific delivery of therapeutic agents (8), which can be especially beneficial
when surgical resection is not indicated due to the metastatic dissemination of cancer cells to other organs, and (ii) hypoinmunogenic properties of MSCs allow sustained release of therapeutic molecules without eliciting unwanted immune response, even in the allogeneic or xenogeneic settings (9). The efficacy and safety of MSCs as delivery vehicles were demonstrated in preclinical models of RCC, where human bone marrow-derived MSCs transduced with adenovirus encoding interleukin-12 (10) or those loaded with conditionally replicative oncolytic adenovirus (11) were utilized to generate tumor site-specific antitumor effects in subcutaneous and orthotopic xenograft models, respectively.

Herpes simplex virus thymidine kinase (HSV-TK)-mediated suicide gene therapy has been widely accepted strategy for cancer. HSV-TK converts the nontoxic nucleoside analogue ganciclovir (GCV) into a toxic triphosphorylated form which can subsequently induce the apoptosis of HSV-TK-transduced cells as well as that of rapidly dividing bystander cells (12). One potential drawback of this strategy is that it fails to destroy slow-proliferating or quiescent tumor cell population, which may cause tumor recurrence. In order to achieve complete eradication of malignant tumor and prevent tumor relapse, HSV-TK/GCV therapy must be used in combination with other therapeutic agents that can eliminate residual tumor cells while enhancing the efficacy of suicide gene therapy.

TNF-related apoptosis-inducing ligand (TRAIL) is a homotrimERIC, type II transmembrane protein that induces apoptosis through both intrinsic and extrinsic death pathway (13). Since TRAIL is known to induce selective apoptosis on transformed cells without affecting normal cells, it has been regarded as a promising candidate for the treatment of cancer. Clinical applications of recombinant TRAIL, however, have been hampered by its short in vivo half-life and intrinsic instability (14). To overcome its limited efficacy, viral
vectors or MSCs were engineered to express TRAIL (15, 16) or agonist monoclonal antibodies targeting TRAIL receptors (17) were developed. One of the potential disadvantages of agonistic antibodies, proposed by a recent study, is that their bivalent feature may reduce therapeutic efficacy and mimicking trimeric structure of native TRAIL is crucial for eliciting full biological activity of agents (18). Another strategy to enhance therapeutic effects of TRAIL is the addition of other treatment modalities for combination therapy. A previous study showed that the combination of agonistic anti-TRAIL receptor antibody with adenovirus-mediated cytosine deaminase/5-fluorocytosine suicide gene therapy produces additive antitumor effects in vivo using human glioma and pancreatic carcinoma xenograft model (19). It was also found that TRAIL can enhance the efficacy of HSV-TK/GCV therapy by augmenting both target and bystander killing effect (20), further supporting the potential clinical utility of combined treatment with HSV-TK/GCV gene therapy and TRAIL-based therapy.

In the present study, we analyzed the therapeutic effects of MSC-mediated combination gene therapy of TRAIL and HSV-TK in murine Renca RCC experimental lung metastasis model. This model was designed to recapitulate post-operative condition in human patients who have not yet developed macroscopically detectable metastatic tumors in the lung - the most common site for RCC metastasis (21). The utilization of MSCs is especially advantageous in such settings, since tumor tropic capacity of MSCs allows them to search and destroy undetectable tumor cells. Furthermore, the use of syngeneic transplantable model enabled proper tumor-host interaction to take place. As RCC (also Renca) is regarded as an immunogenic tumor, the induction of tumor-specific immune responses by MSC therapy was another important parameter for evaluation of therapeutic efficacy. After establishing in vivo
model system, we assessed antitumor effects of engineered MSCs coexpressing dodecameric TRAIL and HSV-TK (MSC/dTRAIL-TK). Dodecameric TRAIL was used in place of conventional trimeric TRAIL based on its superior apoptosis-inducing ability. Engineered MSCs were found to be specifically localized in the lung upon *in vivo* administration, where secreted TRAIL and HSV-TK in conjunction with GCV acts to exert enhanced killing activity against target tumor cells. More importantly, multiple injections of MSC/dTRAIL-TK at a low dose exhibited stronger antimetastatic effects than single injection at a high dose, resulting in 100% survival of tumor-bearing mice after triple injections. Although clinical application of optimistic results from animal models should be taken cautiously, this study demonstrates that multiple injections of MSC/dTRAIL-TK could be an attractive therapeutic approach for completely curing RCC patients with distant metastasis.
Materials and methods

Cells

Rat bone marrow-derived MSCs were prepared as previously described (9). MSCs were cultured in low glucose DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-antimycotic (Invitrogen, Carlsbad, CA). MSCs, in passages 5 through 6, were used for all experiments. RENCA murine renal carcinoma cell line and 4T1 murine breast carcinoma cell line were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 (Welgene, Daegu, Korea) with 10% FBS and antibiotic-antimycotic (Invitrogen).

Adenoviruses and transduction

The DNA sequences encoding dodecameric TRAIL (dTRAIL), tissue plasminogen activator (tPA) secretion signal sequence, sulfactant protein-D (SPD) dodecamerization domain, and extracellular domain of human TRAIL were linked into a DNA cassette, as shown in Fig. 1A. As a control, the isoleucine zipper (ILZ) trimerization domain was used to construct trimeric TRAIL (tTRAIL) (22). To construct a DNA cassette encoding both dTRAIL and HSV-TK, an internal ribosome entry site was inserted between dTRAIL and HSV-TK. All codons of transgenes were optimized to increase expression levels. Recombinant replication-deficient adenoviruses without transgene (rAd/Mock) or encoding enhanced green fluorescence protein (rAd/EGFP), tTRAIL (rAd/tTRAIL), dTRAIL (rAd/dTRAIL), HSV-TK (rAd/TK), or both dTRAIL and HSV-TK (rAd/dTRAIL-TK), were produced using the AdEasyTM Vector System (QBioGene, Carlsbad, CA) as previously described (23) and titrated using the Adeno-X™ rapid titer kit (Clontech, Mountain View, CA). All rAd concentrations used for the transduction of cells are provided as the multiplicity
of infection (MOI) in plaque-forming unit per cells (PFU/cell). For MSC transduction, a
mixture of 50 MOI of rAd and 50 μM of Fe^{3+} was pre-incubated in serum-free medium at
room temperature for 30 minutes, and then infected into MSCs for 30 minutes. Transduction
efficiency of adenovirus in MSCs was about 80%.

**Apoptotic protein expression and apoptosis signaling assay**

To quantify the level of secreted TRAIL from MSC/dTRAIL or MSC/tTRAIL, culture supernatants were analyzed by ELISA using purified anti-human TRAIL antibody (Peprotech, Rocky Hill, NJ) and biotinylated anti-human TRAIL antibody (Peprotech), according to the manufacturer’s instructions. To measure the concentration of TRAIL secreted from MSC/dTRAIL-TK or MSC/dTRAIL upon GCV treatment, culture supernatants were harvested every day and changed with addition of fresh GCV. Collected supernatant were subjected to ELISA as described above.

To analyze the gene expression of HSV-TK, Reverse Transcription-PCR assay was performed using primers for codon-optimized HSV-TK cDNA, (forward, 5’-GCCTTCGACCAGGCCGCTAG-3’ and reverse, 5’-CCATGCCGTGGGGGTCCATCG-3’).

To analyze expression of apoptotic signaling molecules, tumor cells transduced with rAd/mock, rAd/dTRAIL, rAd/Tk, or rAd/dTRAIL-TK were lysed in RIPA buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM DTT, 1 mM PMSF, and 1 mM Na_{3}VO_{4}) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany). After titration, protein expression was analyzed by conventional western blot assay using mouse anti-human TRAIL antibody (Peprotech), caspase-3, caspase-8, PARP, cytochrome c (Cell Signaling
Technology, Beverly, MA), or beta actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro viability assay

To measure the effects of dTRAIL or tTRAIL on MSC viability, MSC were transduced with different doses (0, 10, 50, and 100 MOI) of rAd/mock, rAd/tTRAIL, or rAd/dTRAIL and allowed to grow for three days. Cell viability was measured by a CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega, Madison, WI). Percent survival was calculated as (the absorbance at 490 nm (A$_{490\text{nm}}$) of MSC/dTRAIL or MSC/tTRAIL – A$_{490\text{nm}}$ of blank wells)/(A$_{490\text{nm}}$ of MSC/mock – A$_{490\text{nm}}$ of blank wells) X 100.

To evaluate GCV cytotoxicity on engineered MSCs, various concentrations of GCV (0, 10, 30, and 100 μM) were used to treat MSC/dTRAIL-TK, MSC/dTRAIL, MSC/TK, and MSC/mock. Cell viability was measured at day 0, 1, 3, 5, and 7 and percent survival was calculated as described above.

In vitro co-culture assay

MSC/tTRAIL, MSC/dTRAIL, and MSC/Mock were labeled with 20μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), and then directly co-cultured with RENCA or 4T1 tumor cells at a ratio of 1:1 for 48 h. All floating and adherent cells were harvested, stained with 5 μl of APC-AnnexinV (eBioscience, San Diego, CA) in 100 μl Binding Buffer, and then analyzed by Gallios flow cytometer (Beckman Coulter, Brea, CA) to determine the proportion of apoptotic tumor cells. In a separate experiment, CFSE-labeled MSC/dTRAIL-TK, MSC/dTRAIL, MSC/TK, and MSC/Mock were co-cultured with RENCA cells at a ratio of 1:1 for 48 hours. After further incubation with 100 μM of GCV for
48 hours or 72 hours, the proportion of apoptotic cells was analyzed as described above.

**Animal model and in vivo MSC migration assay**

Female BALB/C (5 –7 week old) mice were purchased from Jackson Laboratory (Bar Harbor, ME). To establish experimental lung metastases, 5 X 10^5 RENCA cells were intravenously injected into the lateral tail vein. To examine the *in vivo* distribution of intravenously-injected MSCs, 5 X 10^5 CFSE-labeled MSCs were injected into the tail vein of naïve control or metastatic tumor-bearing mice. Mice were sacrificed at 24 hours after MSC injection, and then various organs (lung, liver, spleen, heart, kidney, and brain) were isolated. Each organ was minced, treated with collagenase, and prepared for flow cytometric analysis. CFSE-positive cells in each organ were analyzed using a Gallios flow cytometer (Beckman Coulter). To analyze the localization of MSCs in the lung, MSCs were labeled with 5μM CM-DiI (Molecular Probes, Eugene, OR) and 5 X 10^5 cells were intravenously injected into naïve control mice or tumor-bearing mice on day 7 after tumor injection. Mice were sacrificed on day 14 and lung tissues were embedded in paraffin blocks for histological analysis. The labeled MSCs were visualized using a Zeiss Axiovert1135 microscope and AxioVision3 software. For *in vivo* imaging, MSCs were labeled with NEO-LIVE™ Magnoxide 675 (BITERIALS, Seoul, Korea) and intravenously injected into naïve or metastatic tumor-bearing mice on day 7 after tumor injection. Since ventral images can produce abdominal autofluorescence (24), dorsal images of mice were obtained using Maestro™ (CRi, Hopkinton, MA) after being anesthetized. All MSC migration-related assays described above were performed in the absence of GCV administration.

**In vivo antitumor assays**

To investigate antitumor effects of engineered MSCs in metastatic renal cell
carcinoma model, mice were intravenously injected with MSC/Mock, MSC/TK,
MSC/dTRAIL, or MSC/dTRAIL-TK (5 x 10^5 cells) on day 1 or 7 after RENCA cell injection.
Then, 50 mg/kg of GCV was intraperitoneally injected for 7 consecutive days starting on day 0, 1, 2, or 3 after MSC treatment. On day 14 after tumor injection, lungs of tumor-bearing mice were isolated, stained with picric acid, fixed in acetic acid solution, and the number of metastatic tumor nodules on the lung surface was counted. Survival of tumor-bearing mice was monitored up to 100 days post tumor injection.

To investigate the effects of different doses of MSC/dTRAIL-TK on the survival of metastatic tumor-bearing mice, MSC/dTRAIL-TK (5 x 10^5 cells, 1 x 10^6 cells, or 1.5 x 10^6 cells) or MSC/EGFP (5 x 10^5 cells) were intravenously injected into tumor-bearing mice on day 7 after tumor injection. Antitumor effects of sequential therapy were evaluated by repeating second and third injections of 5 x 10^5 cells MSC/dTRAIL-TK at two week interval. GCV was administered for 7 consecutive days starting on day 2 after each MSC/dTRAIL-TK administrations. Survival of tumor-bearing mice was monitored up to 125 days post tumor cell injection.

**Statistics**

All data were expressed as averages ± standard error means. To measure the statistical differences between groups, a two-tailed Student’s t-test was used. For all statistical tests, a P-value of < 0.05 was considered to be statistically significant.
Results

Dodecameric form of TRAIL induces higher levels of tumor cell apoptosis than the trimeric form of TRAIL

To investigate the effects of the dodecamerization of TRAIL on its apoptosis-inducing activity, we generated rAd expressing either dodecameric TRAIL (dTRAIL) or trimeric TRAIL (tTRAIL), as described in Materials and Methods (Fig. 1A). The expressions of dTRAIL and tTRAIL protein from transduced MSCs were confirmed by Western blot (Fig. 1B). When MSCs were transduced with rAd/dTRAIL or rAd/tTRAIL, the inclusion of ferric ion (Fe³⁺) significantly enhanced the expression of both dTRAIL and tTRAIL by 10 to 100 fold. The amount of secreted TRAIL from MSC/dTRAIL in culture supernatant was about 3-fold higher than that from MSC/tTRAIL (Fig. 1C). As the titer of rAd increased, the expression levels of TRAIL also increased, but the viability of MSCs was negatively affected by the viral doses of greater than 100 MOI (Fig. 1D). Decreased MSC viability was unlikely to be caused by high levels of secreted TRAIL, since the extent of decrease in cell survival was comparable between MSC/tTRAIL and MSC/dTRAIL in spite of the difference in their TRAIL induction. This result is supported by a previous observation that MSCs are resistant to TRAIL-mediate apoptosis (25). Based on these results, 50 MOI of rAd in the presence of Fe³⁺ was determined to be the optimal condition for MSC transduction, which did not affect the phenotype of MSCs (Supplementary Fig. 1).

To examine the cytotoxic effects of TRAIL-expressing MSCs on tumor cells, CFSE-labeled MSC/dTRAIL, MSC/tTRAIL, and MSC/mock (control) were co-cultured with RENCA mouse renal cell carcinoma or 4T1 mouse breast cancer cell lines and the proportion
of apoptotic cells was determined. MSC/dTRAIL induced stronger apoptotic responses in RENCA and 4T1 cells, which were known to be sensitive to TRAIL-mediated apoptosis (Fig. 1E-F). MSC/dTRAIL treatment resulted in higher level of apoptosis in RENCA and 4T1 cells than MSC/tTRAIL treatment. It is worth noting that a half-dose of MSC/dTRAIL exerted stronger killing activity against Renca cells than MSC/tTRAIL (Supplementary Fig. 2).

**dTRAIL and HSV-TK exert combinatory effects in apoptosis induction of both transduced MSCs and target tumor cells**

To evaluate the combinatorial effects of dTRAIL and HSV-TK in killing of transduced MSCs as well as bystander tumor cells, we first generated MSCs co-expressing dTRAIL and HSV-TK (MSC/dTRAIL-TK) or HSV-TK alone (MSC/TK). The expression of HSV-TK was confirmed by Reverse Transcription PCR (Fig. 2A). The cytotoxic effects of GCV on engineered MSCs were analyzed by measuring the viability and TRAIL secretion from transduced cells. As shown in Fig. 2B, GCV treatment induced the cell death of TK-expressing MSCs (MSC/dTRAIL-TK and MSC/TK) in a dose-dependent manner, but not in dTRAIL-expressing MSCs. Furthermore, MSC/dTRAIL-TK were found to be more susceptible to GCV-induced apoptosis than MSC/TK. As 100 μM GCV could achieve nearly complete destruction of MSC/dTRAIL-TK after 7 days of treatment (Percentage of remaining MSCs = 1.02 ± 0.58% for MSC/dTRAIL-TK and 6.94 ± 1.74% for MSC/TK), this dosage was selected for the following in vitro co-culture experiment. Consistently, TRAIL secretion from MSC/dTRAIL-TK was significantly reduced by GCV treatment, but not from MSC/dTRAIL (Fig. 2C).

To investigate whether the combination of dTRAIL and HSV-TK could exhibit enhanced apoptogenic effects on bystander tumor cells, CFSE-labeled engineered MSCs
were co-cultured with RENCA cells for 48 hours and treated with 100 μM of GCV for additional 48 or 72 hours. At both time-points, MSC/dTRAIL-TK exhibited stronger apoptotic effects than the sum of effects from MSC/TK and MSC/dTRAIL in RENCA cells (Fig. 3A). It is also worth noting that decreasing the number of MSC/dTRAIL-TK by 75% in co-culture still induced similar level of tumor cell apoptosis to that of MSC/TK (data not shown). A prior study also showed that TK-expressing MSCs could induce cell death of 10 times more tumor cells completely via bystander effect in co-culture assay (26). Fluorescence microscopy also indicated that the highest level of RENCA cell death was observed in co-culture with MSC/dTRAIL-TK (Fig. 3B). To investigate the molecular mechanisms involved in the combinatory action of dTRAIL and HSV-TK, we analyzed the expression levels of apoptosis-related signaling molecules, including caspase-3, caspase-8, cytochrome c, and poly (ADP-ribose) polymerase (PARP). Expression levels of all the molecules tested were significantly increased by co-expression of dTRAIL and HSV-TK (Fig. 3C), which is consistent with the previous report that concerted activation of caspases is responsible for the cooperative antitumor effects between HSV-TK/GCV and TRAIL (20).

**MSC/dTRAIL-TK significantly regresses metastatic tumors and prolongs mice survival, to a greater extent than MSC/dTRAIL or MSC/TK**

To evaluate tumor site-specific localization of MSCs and their duration of residence in vivo, several experimental approaches were utilized. Firstly, CFSE-labeled engineered MSCs (E-MSC, which represents MSC/dTRAIL-TK) were intravenously injected into naïve or tumor-bearing mice at day 1 or 7 after RENCA cell administration. 24 hours after MSC injection, a greater number of engineered MSCs were detected in the lungs of tumor-bearing mice than those of naïve mice (Fig. 4A). In addition, a higher frequency of naïve or
engineered MSCs was detected in the lungs of mice bearing 7-day-old tumors than those with 1-day-old tumors. These results suggest that higher levels of tumor-derived chemokines in the lung may cause more MSCs to be attracted to the tumor sites. Secondly, in vivo imaging analysis was performed to assess the duration of MSC residence in the lung. As shown in Fig. 4B, much stronger fluorescent signals emitted by labeled MSCs were detected in the lungs of tumor-bearing mice than in those of naïve mice throughout the entire observation period. Lastly, to determine whether MSCs could migrate preferentially toward tumor nodules within the lung tissue, the localization of labeled MSCs was investigated by histological analysis on day 7 after MSC injection. Interestingly, a greater number of Dil-labeled MSCs was found in the vicinity of tumor nodule sites compared to non-nodule sites in the lungs of tumor-bearing mice (Fig. 4C). Similar results were reported by a previous report that examined the homing of syngeneic MSCs in mice bearing Renca pulmonary metastasis. In this study, histological analysis of lungs showed that MSCs tended to surround tumor nodules when their diameter is large and infiltrate nodules when the diameter is small (27). Although xenogeneic MSCs were used in our experimental system, cells that reached the tumor nodules seemed to survive longer than those did not, as suggested by the report mentioned above.

Next, we aimed to optimize the time-points for GCV and engineered MSC treatment to maximize the therapeutic efficacy. Firstly, we treated mice with GCV on day 0, 1, 2, or 3 after MSC/dTRAIL-TK injection and analyzed the number of metastatic tumor nodules at 14 days after tumor injection. GCV treatment that was initiated on the same day with MSC/dTRAIL-TK administration induced moderate antitumor effects, presumably due to rapid MSC killing (Fig. 5A). On the other hand, GCV treatment initiated on day 2 maximized the combinatory effects of dTRAIL and HSV-TK, as presented by the fewest number of metastatic nodules,
indicating the dependence of efficacy upon time-point of GCV injection. Thereafter, GCV treatment was initiated on day 2 after MSC/dTRAIL-TK therapy for the following in vivo experiments. Secondly, we compared the anti-metastatic effects of engineered MSCs (MSC/TK, MSC/dTRAIL, MSC/dTRAIL-TK, or MSC/EGFP) administered on day 1 or 7 after RENCA cell injection. Treatment of MSC/TK and MSC/dTRAIL, either on day 1 or 7, elicited similar antitumor activities as shown by about a 50% decrease in the nodule number in comparison to MSC/EGFP-treated mice (Fig. 5B). However, although statistically insignificant, MSC/dTRAIL-TK therapy on day 7 achieved more than 90% regression, showing superior antimetastatic effects compared to day 1 therapy. Therefore, engineered MSC administration was started on day 7 after tumor injection for the following in vivo experiments.

After optimizing GCV and engineered MSC treatment time-points, therapeutic efficacy of engineered MSCs were re-evaluated in the survival experiment. Consistent with the reduction of metastatic tumor nodules, mice treated with MSC/dTRAIL-TK survived for the longest period of time (maximum survival of mice = 38 days for MSC/EGFP, 52 days for MSC/dTRAIL, 58 days for MSC/TK, and 85 days for MSC/dTRAIL-TK). In the end, however, all mice eventually succumbed to their disease.

Multiple injections of MSC/dTRAIL-TK lead to 100% survival of tumor-bearing mice

Although the treatment of MSC/dTRAIL-TK significantly reduced the number of metastatic tumor nodules and prolonged mouse survival, complete remission of metastatic RCC could not be achieved with a single injection of 5 X 10^6 cells. Since the alteration of dose schedule or dose intensity often affects treatment outcome, we evaluated the effects of increasing the dose or frequency of injections on mice survival. Administrations of 2- or 3-
fold higher doses of MSC/dTRAIL-TK (1 x 10^6 and 1.5 x 10^6 cells) induced marginal effects on survival. However, sequential administrations of MSC/dTRAIL-TK (5 x 10^5 cells) in two-week intervals (with double and triple injections) remarkably prolonged the survival. Surprisingly, 50% of tumor-bearing mice treated with two injections of MSC/dTRAIL-TK survived until the end of the study. More importantly, triple injections of MSC/dTRAIL-TK induced a complete cure of metastatic tumor-bearing mice, resulting in 100% survival. When the number of metastatic tumor nodules was counted on day 60 after tumor injection, we found that double injections of MSC/dTRAIL-TK significantly eliminated tumors in the lungs, compared to a single injection, but some tumor nodules still remained in a number of mice (Fig. 6B). On the other hand, triple injections of MSC/dTRAIL-TK completely cleared metastatic tumor nodules in the lungs, which is consistent with the 100% survival rates in the mice that we documented.
Discussion

A major unmet clinical need in the treatment of metastatic RCC is the absence of therapeutic agent that provides durable complete remissions with minimal toxicity. Recent studies have proposed several strategies to achieve complete remission: (i) the selection of therapeutic agents with selective toxicity toward tumor cells, (ii) the identification of the methods of delivering the agents, either in combination or as sequential single agents, and (iii) the optimization of the most effective sequence or combination treatment regimen (28). In this study, we aimed to completely eliminate established tumors in the lung, which is the most common site of metastasis for RCC, by injecting MSCs transduced with a bicistronic adenoviral vector co-expressing dodecameric TRAIL and HSV-TK. Our treatment strategy not only allows tumor site-specific delivery of therapeutic molecules, but also enables the implementation of “sequential combination gene therapy” by repeatedly challenging MSCs expressing two different therapeutic genes. As expected, MSC/dTRAIL-TK exerted more potent antitumor effects than MSC/dTRAIL and MSC/TK upon GCV treatment, both in vitro and in vivo. But surprisingly, complete regression of metastatic RCC was only observed when MSC/dTRAIL-TK was injected repeatedly at a low dose, but not with a single injection at a high dose. To the best of our knowledge, this is the first report to present 100% survival of metastatic tumor-bearing mice by using MSC-based gene therapy alone. Since the majority of metastatic tumors including RCC are resistant to chemo- or radiotherapy, our strategies solely using MSC/dTRAIL-TK, without any combinatory treatment with other agents, can provide a new milestone in the treatment of hard-to-cure metastatic cancer.

Possible explanations for the achievement of complete remission by repeated administrations of MSC/dTRAIL-TK may be summarized as follows: Firstly, the
augmentation of TRAIL-mediated antitumor effects by constructing a dodecameric form, or a 4-trimer form, of conventional soluble TRAIL, partly contributed to long-term remissions. It has been reported that increasing the valency of TRAIL mimics can enhance their binding affinity to TRAIL receptors and apoptogenic potential (29). Our result also agrees with previous work showing that artificial dodecamerization of CD154 (CD40L) using Surfactant Protein D induces superior B cell activation than trimeric CD40L (30). Secondly, the enhanced apoptotic effects by the combination of dTRAIL and HSV-TK/GCV may play an important role in complete eradication of metastatic tumor cells. A prior study proposed that combinatorial action of TRAIL and TK/GCV involves mutual activation of caspases and TK/GCV-mediated mitochondrial amplification of caspase activity. Since toxic GCV triphosphate can be transferred from TK-expressing cells to non-expressing bystander cells via gap junctions, TRAIL improved the killing of bystander cells as well (20). In our in vitro study, the proportion of apoptotic Renca cells induced by MSC/dTRAIL-TK was greater than the sum of those induced by MSC/dTRAIL and MSC/TK, separately. Interestingly, similar results were also found in TRAIL-resistant and taxol-resistant human ovarian cancer cell line A2780-Tax (Annexin V+ population at 24 hours post GCV treatment = 7.66 ± 0.55% for MSC/mock, 12.81 ± 3.07% for MSC/dTRAIL, 41.68 ± 1.89% for MSC/TK, and 98.00 ± 0.11% for MSC/dTRAIL-TK, unpublished data), suggesting that TRAIL resistance could be overcome by combination therapy of TRAIL and HSV-TK/GCV in selected cancer cell lines. Thirdly, the capability of dTRAIL and HSV-TK/GCV to target different populations of heterogeneous RCC cells may also aid in accomplishing durable complete remission. It is well-established that tumors consist of heterogeneous population of cells having diverse molecular profiles, proliferative and metastatic potential, differentiation capacity, and
susceptibility to chemo- and radiation therapy (31). Among these cells, the capability to initiate tumor formation and sustain tumor growth resides in a small subset of quiescent, slow-growing populations known as cancer stem cells (CSCs) (32). A recent study has shown the existence of CSCs in Renca cells by isolating side population (SP) cells (33). Since HSV-TK/GCV therapy only eliminates rapidly proliferating tumor cells, residual slow-proliferating cells may repopulate to form recurrent tumor. Indeed, SP cells were found to be more resistant to HSV-TK/GCV treatment than non-SP cells in human glioblastoma (34). On the other hand, SP cells were more sensitive to TRAIL than non-SP cells in human colon cancer, due to the increased expression of c-Myc and consequent upregulation of TRAIL receptor DR4 (35, 36). Similarly, radioresistant CSC-like oesophageal cells exhibited a higher susceptibility to TRAIL therapy than parental cells (36). These results suggest that the combination therapy of TRAIL and HSV-TK/GCV is an optimal strategy to simultaneously target SP and non-SP cells. Fourthly, the induction of stronger tumor-specific T cell responses by the combination of dTRAIL and HSV-TK/GCV might have contributed to the long-term remission (IFN-γ spots/10^6 splenocytes = 81 ± 15 for MSC/EGFP, 124 ± 7 for MSC/dTRAIL, 173 ± 17 for MSC/TK, and 222 ± 20 for MSC/dTRAIL-TK). The previous evidence suggests that TRAIL-mediated tumor destruction increases the amount of antigens that could be delivered into the cross-presentation pathway, ultimately leading to enhanced antitumor immunity (4). HSV-TK/GCV therapy is also capable of inducing potent T cell and NK cell responses (37). As immunogenic properties of RCC renders tumor cells relatively sensitive to immunological attack, the development of strong immune responses may play a role in complete regression. Lastly, but most importantly, the optimization of biological dose and schedule for MSC/dTRAIL-TK treatment contributed a great deal to the accomplishment
of durable complete remission. The optimal time points for *in vivo* application of GCV and
MSCs were empirically determined to provide the maximum therapeutic efficacy. We then
evaluated the effects of biological doses and frequency of administration on tumor regression.
No significant increase in survival was observed after a single injection of MSC/TRAIl-TK
at two or three times higher doses. Repeated administration of small divided doses of
MSC/TRAIl-TK, however, markedly improved mice survival, resulting in 50% survival
after two injections and 100% survival after three injections. Multiple injections of small
divided doses of agents were as effective as or even more advantageous than single injection
in some occasions. For example, frequent smaller doses of bevacizumab exerted similar
modulatory effects with a single large injection on vascular endothelial growth factor-induced
vascular changes in retinal neovascular model (38). It was also reported that low-dose
repeated injections of adriamycin was more effective than a high-dose single injection in
terms of improving the survival of tumor-bearing mice while lowering the drug toxicity (39).
Sustained release of therapeutic molecules in the tumor microenvironment and more frequent
exposure to GCV seemed to be crucial for the achievement of complete remission in our
experimental system. The potency of sequential suicide gene therapy was also assessed in a
recent study, where repeated injections of human adipose-derived MSCs expressing cytosine
deaminase::uracil phosphoribosyltransferase (with intracerebroventricular 5-FC
administration) achieved 88% survival of rats bearing intracerebral glioblastoma (40).

In conclusion, sequential administration of MSCs co-expressing dTRAIL and TK is a
safe and potent strategy for accomplishing long-term remissions of metastatic RCC. The
application of syngeneic or allogeneic MSCs, which are more often used in clinic, may
further potentiate the therapeutic efficacy of MSC/dTRAIL-TK as more MSCs would survive
at the tumor site to increase the availability of therapy. Moreover, the incorporation of HSV-
TK/GCV system enables efficient elimination of MSCs, lowering the risk of malignant
transformation of infused MSCs. Overall, our findings propose a novel therapeutic approach
to achieve durable complete remission in metastatic RCC.
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References


Figure legends

Figure 1. Relative expression levels and apoptotic potential of trimeric and dodecameric TRAIL expressed by engineered MSCs. (A) Schematic diagram of DNA constructs encoding EGFP, tTRAIL, dTRAIL, dTRAIL-TK, or TK. tTRAIL: Trimeric TRAIL, dTRAIL: dodecameric TRAIL, tPA-S: tissue plasminogen activator signal sequence, ILZ: isoleucine zipper, SPD: sulfactant protein D, IRES: internal ribosome entry site, HSV-TK: herpes simplex virus thymidine kinase, EGFP: enhanced green fluorescence protein (B) TRAIL expression of MSC/tTRAIL and MSC/dTRAIL was analyzed by western blot assay using cell lysates. (C) MSCs were transduced with increasing titers of rAd/tTRAIL or rAd/dTRAIL in the presence or absence of Fe^{3+} (50 μM) for 30 minutes. TRAIL expression level was analyzed by ELISA, using supernatant, at 24 hours after transduction. **p < 0.01. (D) At three days after transduction of MSCs with rAd/tTRAIL or rAd/dTRAIL, MSC cell viabilities were determined by CellTiter 96 Aqueous One Solution cell proliferation assay. Data represent averages ± SEM of three independent experiments. (E and F) MSC/tTRAIL or MSC/dTRAIL were cocultured with Renca (E) or 4T1 cells (F) at a ratio of 1:1 for 48 hours. The percentages of apoptotic cells were then quantified by Annexin V staining. Data represent average percentages ± SEM of three independent experiments.

Figure 2. Effects of GCV treatment on cell death and TRAIL expression in engineered MSCs. (A) Expression of HSV-TK in engineered MSCs was analyzed by Reverse transcription PCR at 48 hours after transduction of MSCs. (B) Various concentrations (0, 10, 30, or 100 μM) of GCV were treated to engineered MSCs, and then cell viability was measured by CellTiter 96 Aqueous One Solution cell proliferation assay. (C) TRAIL
secretion by engineered MSCs was analyzed at indicated time-points after GCV treatment using ELSIA assay. Data represent average ± SEM of four independent experiments.

Figure 3. Combinatory effect of dTRAIL and HSV-TK on apoptotic induction in Renca cells. (A) Renca cells were co-cultured with engineered MSCs labeled with CFSE at a ratio of 1:1 for 48 hours and then treated with 100 μM of GCV. At 48 hours or 72 hours after GCV addition, all floating and attached cells were harvested. The percentages of apoptotic cells were quantified by Annexin V staining. Data represent averages ± SEM of four independent experiments. (B) Renca cells were labeled with Calcein-AM (green) and co-cultured with CM-DiI-labeled MSCs (red) for 48 hours, and treated with 100 μM GCV for another 48 hours. Labeled cells were visualized by fluorescence microscopy. Scale bar: 100 μm. (C) Renca cells transduced with various rAd constructs were incubated in the presence of 100 μM GCV for 48 hours. Expression levels of apoptosis-related molecules (such as caspase-3, caspase-8, PARP, and cytochrome c) in Renca cells were analyzed using western blot. β-actin was used as an internal control.

Figure 4. Tumor-tropism of naive or engineered MSCs. (A) At 1 or 7 days after intravenous injection of Renca cells into BALB/C mice, CFSE-labeled naïve or engineered MSCs expressing dTRAIL and TK (E-MSC) were intravenously injected into naïve or tumor-bearing mice. Percentages of CFSE-positive MSCs in the lung or liver tissues were analyzed by FACS at 24 hours after MSC injection. Data represent mean values observed in two separate experiments. **p < 0.01. (B) On day 7 after intravenous injection of Renca, magnoxide 675-labeled MSCs were intravenously injected. The migration of MSCs was
analyzed by *in vivo* imaging system under fixed exposure time at the indicated time points after MSC. Red arrows indicate MSCs detected in the lung. On day 4 and 7 after MSC injection, lungs were harvested from naïve and tumor-bearing mice and labeled MSCs were visualized. (C) CM-Dil-labeled MSCs (red) were intravenously injected into naive mice or mice bearing 7-day-old tumor. Lungs were isolated and prepared for histological analysis at 7 days after MSC injection. Images with CM-Dil-labeled MSCs (red) and DAPI positive cells (blue) were examined by fluorescence microscopy. Tumor nodules are indicated by white dashed line. Corresponding hematoxylin and eosin-stained sections of each fluorescent images were also presented. T: tumor nodule; scale bar: 100 μm.

**Figure 5. Relative anti-metastatic effects of engineered MSCs.** (A) Engineered MSCs were intravenously injected on day 7 after tumor injection, and then GCV treatment was initiated on day 0, 1, 2, or 3 after MSC injections for 7 consecutive days. The numbers of metastatic tumor nodules in the lung were counted on day 14 after tumor injection (n = 10). (B) Engineered MSCs were intravenously injected on day 1 or day 7 post tumor injection (PTI), and then GCV was treated for 7 days, starting on day 2 after MSC injection. The number of metastatic tumor nodules in the lung was counted on day 14 after tumor injection (n = 11). Representative pictures of lungs (therapy on day 7 PTI) are shown. **p < 0.01. (C) Engineered MSCs were injected on day 7 after tumor injection, and then GCV was treated for 7 days, starting on day 2 after MSC injection. The survival rate of tumor-bearing mice was monitored up to 100 days post tumor injection (n = 9). These results are representative of three independent experiments.
Figure 6. Therapeutic effects of multiple injections of MSC/dTRAIL-TK on the survival of metastatic tumor-bearing mice.

(A) MSC/dTRAIL-TK or MSC/EGFP (5 x 10⁵ cells) were injected into tumor-bearing mice once (single) or repeatedly (double or triple) over an interval of two weeks, starting on day 7 after tumor injection (n = 10). In addition, different doses of MSC/TRAIL-TK (5 x 10⁵, 1 x 10⁶, 1.5 x 10⁶ cells) were injected on day 7 after tumor injection (n = 6). GCV was treated daily for 7 days after each MSC injection. The survival rate of tumor-bearing mice was monitored up to 125 days post tumor injection. White arrows indicate the time-points for MSC injection. (B) Representative pictures of the lungs isolated on day 60 are shown. Black arrows indicate tumor nodules. These results are representative of three independent experiments.
Figure 1. Relative expression levels and apoptotic potential of trimeric and dodecameric TRAIL expressed by engineered MSCs.
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Figure 2. Effects of GCV treatment on cell death and TRAIL expression in engineered MSCs

A

B

MSCiTRAIL-TK

MSC/TK

MSCiTRAIL

Cell viability (% of control)

0 10 20 30 40 50 60 70 80 90 100

GCV (µg)

Day 1

Day 2

Day 3

Day 4

C

MSCiTRAIL-TK

MSCiTRAIL

Secreted TRAIL, ng/mL

0 10 20 30 40 50 60 70 80 90 100

Secreted TRAIL, ng/mL

Day 1

Day 2

Day 3

Day 4
Figure 3. Combinatory effect of dTRAIL and HSV-TK on apoptotic induction in Renca cells
Figure 4. Tumor-tropism of naïve or engineered MSCs

A

Lung

% CFSE-labeled MSCs in lungs

- Naive
- Tumor-bearing

Injected cells
- E-MSC
- MSC

Injection time point
(day after tumor challenge)
- Day 1
- Day 7

Liver

% CFSE-labeled MSCs in livers

- Naive
- Tumor-bearing

Injected cells
- E-MSC

Injection time point
(day after tumor challenge)
- Day 1
- Day 7

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Figure 4. Tumor-tropism of naïve or engineered MSCs

B

1 hour 6 hours 1 day 2 days 3 days 4 days 7 days

Negative control

Naïve mice + MSCs

Tumor-bearing mice + MSCs

4 days 7 days

Negative control

Naïve mice

Tumor-bearing mice
Figure 4. Tumor-tropism of naïve or engineered MSCs
Figure 5. Relative anti-metastatic effects of engineered MSCs
Figure 6. Therapeutic effects of multiple injections of MSC/dTRAIL-TK on the survival of metastatic tumor-bearing mice.
Complete regression of metastatic renal cell carcinoma by multiple injections of engineered mesenchymal stem cells expressing dodecameric TRAIL and HSV-TK

Sae Won Kim, Soo Jin Kim, Sang-Hoon Park, et al.

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