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Review

Cell and gene therapy using mesenchymal stem cells (MSCs)

Keiya Ozawa [a,b,*], Kazuya Sato [a], Iekuni Oh [a], Katsutoshi Ozaki [a], Ryosuke Uchibori [b], Yoko Obara [a,b], Yuji Kikuchi [a,b], Takayuki Ito [b], Takashi Okada [b,1], Masashi Urabe [b], Hiroaki Mizukami [b], Akihiro Kume [b]

[a] *Division of Hematology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan*
[b] *Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan*

Abstract

Mesenchymal stem cells (MSCs) are considered to be a promising platform for cell and gene therapy for a variety of diseases. First, in the field of hematopoietic stem cell transplantation, there are two applications of MSCs: 1) the improvement of stem cell engrafting and the acceleration of hematopoietic reconstitution based on the hematopoiesis-supporting ability; and 2) the treatment of severe graft-versus-host disease (GVHD) based on the immunomodulatory ability. Regarding the immunosuppressive ability, we found that nitric oxide (NO) is involved in the MSC-mediated suppression of T cell proliferation. Second, tumor-bearing nude mice were injected with luciferase-expressing MSCs. An in vivo imaging analysis showed the significant accumulation of the MSCs at the site of tumors. The findings suggest that MSCs can be utilized to target metastatic tumors and to deliver anti-cancer molecules locally. As the third application, MSCs may be utilized as a cellular vehicle for protein-supplement gene therapy. When long-term transgene expression is needed, a therapeutic gene should be introduced with a minimal risk of insertional mutagenesis. To this end, site-specific integration into the AAVS1 locus on the chromosome 19 (19q13.4) by using the integration machinery of adeno-associated virus (AAV) would be particularly valuable. There will be wide-ranging applications of MSCs to frontier medical treatments in the near future.
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Keywords: Cancer gene therapy; GVHD; Mesenchymal stem cells; Site-specific integration; Tumor targeting

1. Introduction

In bone marrow, there are different types of tissue stem cells (adult stem cells); i.e. hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs account for a small population of cells in bone marrow as a non-hematopoietic component with the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells [1]. Recent studies demonstrated that MSCs are capable of supporting hematopoiesis and of regulating immune response [2]. In addition, since MSCs can be readily isolated and expanded *in vitro*, they are expected to be a source of cell therapy. Interestingly, MSCs have the ability to accumulate at the site of: i) tissue/organ damage; ii) inflammation; and iii) cancer when administered *in vivo*. Therefore, MSCs can be utilized for: i) regenerative therapy; ii) treatment of graft-versus-host disease (GVHD) and Crohn disease; and iii) platform of cancer gene therapy (targeted delivery of anti-cancer agents). Another unique feature of MSCs is little or low immunogenicity due to the lack of expression of co-stimulatory molecules. This phenomenon makes it possible to administer MSCs without HLA matching for cell therapy. A single lot of expanded MSCs from one healthy donor can be utilized for treatment of many patients. Although clinical applications of MSCs have been conducted for the suppression of severe acute GVHD in allogeneic stem cell transplantation [3,4] and for regenerative therapy [5,6], molecular mechanisms underlying the biological effects of

* Corresponding author. Jichi Medical University, Division of Hematology, Department of Medicine, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi, 329-0498, Japan. Tel.: +81 285 58 7353; fax: +81 285 44 5258.
E-mail address: kozawa@ms2.jichi.ac.jp (K. Ozawa).
[1] Present address: Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan.

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MSCs remains obscure. Finding key molecules for differentiation, immunosuppression, and hematopoietic support of MSCs would be valuable for further augmenting the efficacy of MSCs in a wide range of clinical applications. In this regard, development of the technology for genetic manipulation of MSCs is also important research project. Site-specific integration of a therapeutic gene into a safe locus in the genome should be investigated from the safety standpoint.

2. Microarray analysis of genes responsible for differentiation of mesenchymal stem cells

Genes regulating the differentiation of MSCs remain obscure and it is technically difficult to do high-throughput analysis using primary MSCs, because such cells contain heterogeneous populations. To overcome the problems related to the heterogeneity of primary MSCs, we utilized MSC-like cell lines. It has been shown that 10T1/2 cells, derived from C3H mouse embryo cells, differentiate into adipocytes, osteocytes, and chondrocytes with a treatment of 5-azacytidine. We previously established two sub-lines from 10T1/2, designated as A54 for a preadipocyte cell line and M1601 for a myoblast cell line [7]. Under appropriate culture conditions, A54 and M1601 cells terminally differentiate into adipocytes and myotubes, respectively, while parental 10T1/2 cells remain undifferentiated under the same culture conditions. Therefore, 10T1/2 cells can be utilized as a model of MSCs, and A54 and M1601 are used as committed mesenchymal progenitors. Gene expression profiles of these cell lines were compared by microarray analysis before and after differentiation.

Each of parent 10T1/2, A54, and M1601 cell lines showed a distinctive and unique gene expression profile despite morphological similarity (Fig. 1) [8]. Parental 10T1/2 cells had 105 elevated genes including ones encoding Activin, Dlk, Nov, Grb10, p15, and many functionally unknown molecules. Dlk and Nov are known to be involved in Notch signaling pathway and were reported to have the ability to inhibit differentiation into adipocytes and osteoblasts [9]. In preadipocyte A54 cells, 201 genes were up-regulated, including genes known to be involved in adipocyte differentiation such as genes encoding C/EBPα, C/EBPδ, PPAR-γ, PAI-I, and Frizzled-1 [10]. Myoblasts M1601 cells showed 137 up-regulated genes, including ones related to skeletal muscle differentiation such as genes encoding MyoD, MLC1F, α-skeletal actin, myosin heavy chain, and myosin light chain [11] as well as genes related to cardiac muscle differentiation such as genes encoding α-cardiac actin, cardiac troponin C, and troponin T2 [12].

Previous studies have shown that preadipocytes have a higher ability to support hematopoiesis than other kinds of stromal cell components *in vitro* [12,13]. Our results of gene expression profile revealed up-regulation of critical cytokines for hematopoiesis such as SCF and SDF-1 in preadipocyte A54 cells. In addition, many chemokines, such as CXCL-1 and CCL-7, were also up-regulated. Since Ang-1 was reported to be indispensable for the self-renewal of hematopoietic stem cells [14], we performed real-time PCR analysis of Ang-1 along with SCF, SDF-1, CEBP-δ, IGF-1, and CXCL-1. The expression of these genes was highest in A54 cells among the three cell lines. Moreover, protein expression of Ang-1 was only detected in A54 among three cell lines and the level of this protein decreased after adipocyte differentiation.

To examine the effects of these three lines on hematopoiesis, we co-cultured mouse hematopoietic stem cell fraction with these three stromal cell lines. The cells in Lin(−)Sca-1(+) fraction were plated on 10T1/2, A54, or M1601 cells.

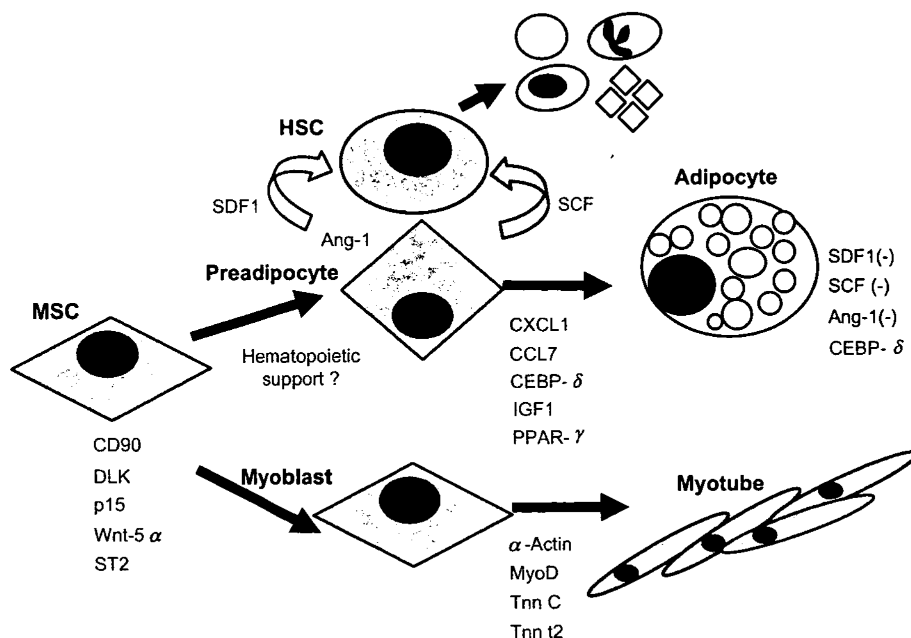

Fig. 1. Proposed model for the hierarchy of the bone marrow stromal system [8].

After 6 days of co-culture, hematopoietic progenitors were detected only on the A54 cells. These results suggest that only A54 cells have the ability to support hematopoietic cell growth among these three cell lines, consistent with the previous report. Hematopoietic cell proliferation was not observed on the layer of the terminally differentiated A54 adipocytes, suggesting that A54 cells lose the ability for hematopoietic cell support after adipocyte differentiation. To understand the molecular mechanisms of this observation, we examined the expression levels of SCF, SDF-1, and Ang-1 during adipocyte differentiation by RT real-time PCR. The expression levels of Ang-1 and SCF decreased immediately after the induction of adipocyte differentiation, and that of SDF-1 decreased gradually. In contrast to this, the level of adipocyte differentiation marker, CEBP-δ, was unchanged.

The analysis of functionally unknown molecules is currently underway. In addition, cell-to-cell contact is also believed to be crucial in the interaction between hematopoietic stem cells and MSCs. We are currently investigating the cellular and molecular events in the interactive communication between hematopoietic stem cells and MSCs.

3. Nitric oxide (NO) plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

There is a case report of severe steroid-resistant GVHD after bone marrow transplantation, in which intravenous infusion of MSCs greatly improved clinical manifestations [3]. Moreover, multi-institutional clinical trial of MSC-treatment of severe grade III–IV acute GVHD in Europe revealed very high overall response rate (about 70%) (Le Blanc et al., ASH meeting 2006). The molecular mechanisms by which MSCs suppress T-cell proliferation are complicated, and whether a soluble factor plays a major role remains controversial. Transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E_2 (PGE_2) have been reported to mediate T-cell suppression by MSCs [15–17]. In addition, some reports have shown that a soluble factor is the major mediator of suppression, whereas some reports have demonstrated that T-cell-MSC contact is required for this suppression.

We also investigated the molecular mechanisms using primary murine MSCs, and focused on nitric oxide (NO), because it is known to inhibit T-cell proliferation. NO is produced by NO synthases (NOSs), of which there are 3 subtypes; i.e. inducible NOS (iNOS), endothelial NOS, and neuronal NOS. It has been known that macrophages suppress T-cell proliferation, and that this suppression is caused by NO-mediated inhibition of Stat5 phosphorylation [18]. We investigated whether MSCs can also produce NO and whether NO is involved in their ability to suppress T-cell proliferation [19].

T cells proliferated in response to PMA and ionomycin, which act downstream of the T-cell-receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. Such T-cell proliferation was suppressed by the presence of MSC, suggesting that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx. The expression of the activation markers CD25 and CD69 on CD4 or CD8 T cells did not change even in the presence of MSCs. MSCs suppressed the production of IFN-γ but not IL-2.

Although T cells from $Stat5^{-/-}$ mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25. Because this phenotype is similar to the status of activated T cells in the presence of MSCs, we hypothesized that MSCs suppress Stat5 phosphorylation. Indeed, Stat5 phosphorylation in activated T cells was diminished in the presence of MSCs. We found that MSCs caused a significant and cell-dose-dependent production of NO only when co-cultured with activated T cells. The induction of iNOS was readily detected in MSCs but not in T cells. RT-PCR and Western blot analysis detected iNOS expression in MSCs cocultured with activated splenocytes but not in MSCs or splenocytes when cultured alone. The immunofluorescence studies showed that iNOS was exclusively expressed in $CD45^-$ adherent cells, which correspond to MSCs, but not in $CD45^+$T cells. Next, we investigated the effects of *N*-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells. Importantly, L-NAME restored T-cell proliferation and Stat5 phosphorylation, indicating that NO is involved in the inhibition of T-cell proliferation and Stat5 phosphorylation. Moreover, MSCs from inducible $NOS^{-/-}$ mice had a reduced ability to suppress T-cell proliferation.

In the presence of direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation. In contrast, both NO production and T-cell suppression were reduced in a transwell system, in which T cells were separated from MSCs by a 1-μm-pore membrane. There are two possible explanations for the difference in T-cell suppression between the presence and absence of the transwell system. First, the amount of NO produced in the transwell system was lower than that in the presence of direct interaction. This finding suggests that direct interaction is critical for efficient production of NO as well as for strong suppression of T-cell proliferation. A second possible explanation is that, because NO is highly unstable, it can lose its activity before it reaches T cells in the transwell system.

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs, we compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-methyl-DL-tryptophan (1-MT: inhibitor of IDO) or an anti-TGF-β-neutralizing antibody restored T-cell proliferation as effectively as L-NAME; however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression.

In summary, our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through inhibition of Stat5 phosphorylation was supported by the following facts: (1) NO was readily detected in the medium in the co-culture of MSCs and activated T cells; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from $iNOS^{-/-}$ mice had markedly

reduced ability to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS expression was detected only in MSCs co-cultured with activated T cells.

In our scenario (Fig. 2), when MSCs are administered to the patients with severe acute GVHD, MSCs are considered to accumulate at the site of inflammation. Upon interaction with activated T cells, MSCs express iNOS and produce NO, which suppresses T-cell proliferation via inhibition of STAT5 phosphorylation. Systemic adverse effects of NO do not occur due to local production of NO with very short half-life. This is a very important point, because conventional treatment of acute GVHD causes severe systemic immunosuppression, which sometimes leads to life-threatening infections. Since MSC treatment causes just local immunosuppression, it should be much safer.

4. Interferon-γ and NF-κB mediate nitric oxide production by mesenchymal stem cells

Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation. Therefore, we investigated whether mouse bone-marrow-derived MSCs and the 10T1/2 cell lines have the same effect on Th1 and Th2. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions, where NO production was highly induced in the presence of MSCs in Th1 but it was only minimally induced in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1, produce high levels of NO in Th1 condition. These results suggest that NO also plays a major role in the preferential suppression of Th1 proliferation by MSCs.

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that support Th2 differentiation, anti-IFN-γ antibody and IL-4, were investigated. As a result, anti-IFN-γ antibody clearly inhibited the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN-γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated T cells had the ability to induce NO production by MSCs. IFN-γ is critical for NO production; however, in a T cell-free environment, IFN-γ alone does not induce NO production from primary MSCs. IFN-γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs, suggesting that both the IFN-γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. The addition of flagellin induced NO production in combination with IFN-γ. While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO. Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN-γ signal. In addition to these factors, IL-1β and TNF-α induce NO when provided in combination with IFN-γ. As NF-κB is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1β, and TNF-α, we hypothesized that activation of NF-κB is required for NO induction by MSCs. Bay-11-7085, a specific inhibitor of NF-κB, suppressed induction of iNOS in MSCs, thus suggesting that NF-κB is involved in NO production by MSCs as well as IFN-γ [20].

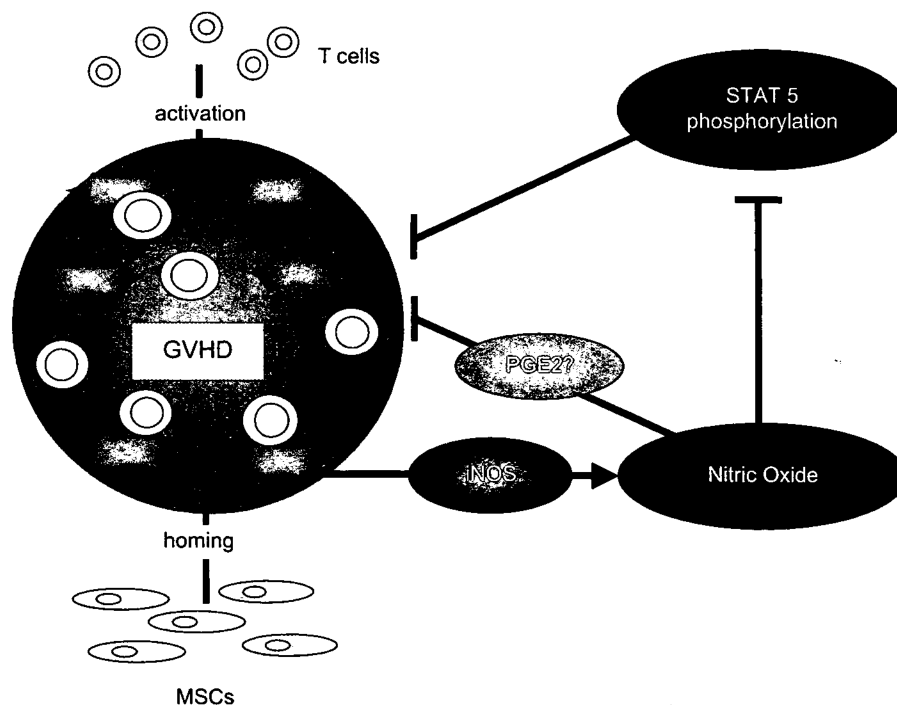

Fig. 2. MSC treatment of acute GVHD and the molecular mechanisms of T-cell suppression. MSCs are considered to accumulate at the site of inflammation and systemic adverse effects may not appear due to the local production of NO, which has very short half-life.

5. Retroviral vector-producing mesenchymal stem cells for tumor tracking and therapeutic gene amplification in suicide cancer gene therapy

MSCs are known to have a tendency to accumulate at the site of tumors, and therefore can be utilized as a platform for targeted delivery of anti-cancer agents [21–23]. The MSC-based targeted cancer gene therapy can enhance the therapeutic efficacy, because MSCs are considered to reach tumors including metastatic lesions and to deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the anti-cancer agents act locally at the site of tumors without elevating their systemic concentrations. We developed genetically-modified MSCs that produce retroviral vectors encoding HSV*tk*, aiming at augmenting therapeutic efficacy of systemic suicide cancer gene therapy (Fig. 3). The tumor tropism and anti-tumor effects of vector-producing MSCs (VP-MSCs) were examined by intravascular injection in tumor-bearing nude mice. MSCs isolated from the bone marrow of SD rats were transfected with plasmid DNA expressing luciferase alone (=non-VP-MSCs) or whole retroviral vector components (LTR-Luc or LTR-HSV*tk* with Gag-pol and VSV-G) (=VP-MSCs) by nucleofection. To assess tumor tropism of MSCs, nude mice were subcutaneously inoculated with 9 L rat glioma cells or Rat-1 fibroblasts, and were subsequently injected with luciferase-expressing MSCs through the left ventricular cavity. The transgene expression was periodically traced by using an *in vivo* imaging system. As a result, the transgene expression accumulated at the site of subcutaneous 9 L tumors, but undetectable at the site of Rat-1 fibroblasts. In addition, the injection of luciferase-expressing VP-MSCs caused much stronger signal of bioluminescence at the site of 9 L tumors compared with luciferease-expressing non-VP-MSCs. Immunostaining study showed that luciferase-positive cells (injected MSCs and transduced glioma cells) were detected at the periphery of tumors. To evaluate the therapeutic efficacy, tumor-bearing nude mice were treated with non-VP-MSCs or VP-MSCs combined with HSV*tk*/GCV system and then the size of subcutaneous tumors was periodically measured. In this model experiments, tumor growth was more efficiently suppressed by injecting VP-MSCs compared with non-VP-MSCs (Uchibori R, et al.: manuscript in preparation). This study suggests the effectiveness of VP-MSCs in suicide cancer gene therapy. The therapeutic benefit of this strategy should be further examined in orthotopic and metastatic tumor models.

6. Site-specific insertion of a therapeutic gene into the AAVS1 locus (19q13.4) in human mesenchymal stem cells by using adeno-associated virus integration machinery

Hematopoietic stem cells, ES cells, and MSCs are attractive targets for gene therapy and regenerative medicine, since they replicate themselves and differentiate into various cell lineages. To introduce genes in these stem cells, it is especially important to utilize a system that results in a minimal risk of insertional mutagenesis. To date, only one animal virus, the adeno-associated virus (AAV), is able to integrate into a defined site in human chromosome, AAVS1 (19q13.4), which is mediated by the activity of specific replicase/integrase protein, Rep. The Rep78 or Rep68 protein recognizes the GAGC motif on the viral inverted terminal repeat (ITR) sequence and a similar motif in AAVS1, leading to the site-specific integration of the AAV genome.

We and others have reported that a plasmid transfection system utilizing AAV derived components, the *rep* gene and ITR, could integrate the gene of interest preferentially into AAVS1 in epithelial or adherent cells (e.g., 293, HeLa, Huh-7 cells) [24–26]. Our system uses two plasmids, one harboring the transgene cassette flanked by the ITR sequences, and the other for *rep* expression, allowing only plasmid DNA harboring the ITR to integrate into the AAVS1 locus. In addition, this system can deliver DNA segments larger than the 4.5-kb packaging limit of AAV. As a first step toward establishing a method capable of integrating therapeutic DNA into the AAVS1 locus in MSCs, we tested this strategy in KM-102 cells, a cell line derived from human marrow stromal cells. KM-102 cells were co-transfected with a bicistronic plasmid containing a humanized GFP gene and a blasticidin S resistance gene (*bsr*) between the ITRs and a Rep68 plasmid. After transfection, single cell clones were grown in the presence of

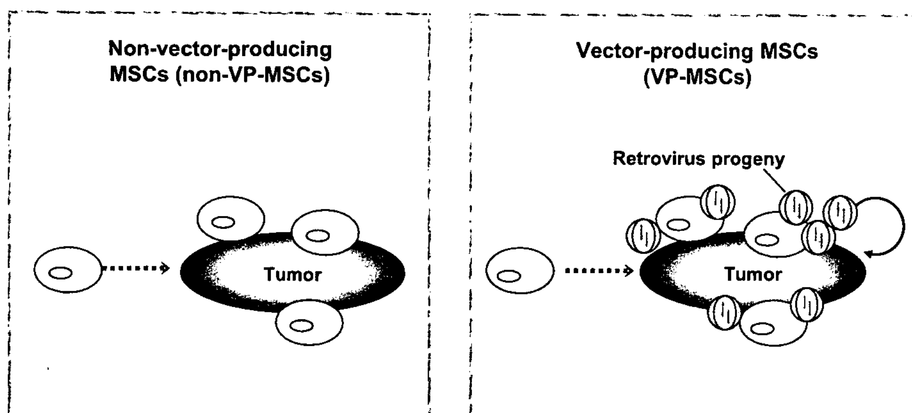

Fig. 3. Development of vector-producing tumor-tracking MSCs to augment suicide cancer gene therapy.

blasticidin S. Southern blot analysis of their genomic DNA revealed that three out of eight blasticidin S resistant clones showed site-specific integration of transgene into the AAVS1 site and that these clones had the GFP gene only at AAVS1. These results indicated that foreign DNA linked with ITR sequence could be targeted specifically into AAVS1 in KM-102 cells.

It is reported that the genome of myosin binding subunit 85 (MBS85) overlaps with the AAVS1 site [27]. To identify the junction between the transgene plasmid and the AAVS1 site, PCR was conducted using a transgene- and an AAVS1-specific primers. In two of the three clones the integration site was identified. In one clone the GFP gene was inserted at the first intron of MBS85 gene. The other clone had insertion of the GFP gene upstream of the first exon. Quantification of mRNA for MBS85 by real time PCR showed that the mRNA level decreased in these two KM-102 clones. The MBS85 is involved in the assembly of actin cytoskeleton. Although the outcome of allelic disruption of the MBS85 genome should be carefully evaluated, the system for AAVS1-specific integration of therapeutic DNA using AAV integration machinery is particularly valuable for *ex vivo* gene therapy applications for stem cells, such as ES cells and MSCs. For additional readings on the use of bone marrow cells for the treatment of autoimmunity, the reader is referred to companion papers published herein in this special issue of the Journal of Autoimmunity [28–38].

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Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy

Takashi Okada[1], Keiya Ozawa[2]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan,* [2]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Japan*

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1. ABSTRACT

Suicide cancer gene therapy with retroviral vector-producing cells was in the way of an adjuvant to the surgical resection of recurrent glioblastoma, although any benefit appeared to be marginal. It is likely that this therapeutic approach may have better outcomes if the vectors and transgenes are delivered more efficiently to the tumor cells. We have shown previously that tumor cells engineered by adenovirus-retrovirus hybrid vectors to produce retroviral progeny destroy satellite tumor cells. Whether the systemic delivery of vector-producing cells can effectively treat aggressive tumors remains to be determined. Effective retroviral vector delivery vehicles may be multipotent mesenchymal stromal cells (MSCs), which have been shown to home to tumor cells *in vivo* and deliver cancer-killing gene or immune products with minimal host rejection. Therefore, it may be possible to transduce tumors with recombinant progeny vectors delivered by MSCs. This may be particularly suitable for treating diffuse cancers like glioblastoma multiforme. While this strategy remains to be tested in various orthotopic or metastatic tumor models, it has the potential to greatly improve the outcome of suicide gene therapy.

2. SUICIDE CANCER GENE THERAPY USING VECTOR-PRODUCING CELLS

A gene therapy against glioma has been developed in which a cell line that continuously secretes a retroviral vector is implanted into brain tumors (1). The vector, which expresses the herpes simplex thymidine kinase (HSV-*tk*) transgene, "infects" the local tumor cells, which then become susceptible to tumoricidal metabolites generated by HSV-*tk*-mediated activation of the prodrug ganciclovir (GCV). The therapeutic value of this technique appeared to be enhanced by a bystander effect wherein the transduced tumor cells communicate the apoptosis signal to neighboring cells. However, while this cancer gene therapy system was shown to have some clinical benefit, its efficacy was limited due to the poor efficiency of gene transfer (2). To improve the therapeutic potential of this system, it is necessary to enhance (a) the efficiency of therapeutic gene delivery *in vivo* and (b) the stability of the vector-producing cells.

With regard to therapeutic gene delivery *in vivo*, we have previously described a hybrid vector system where adenoviral vectors are used to deliver retroviral vector andpackaging proteins into cells (3). This system benefits

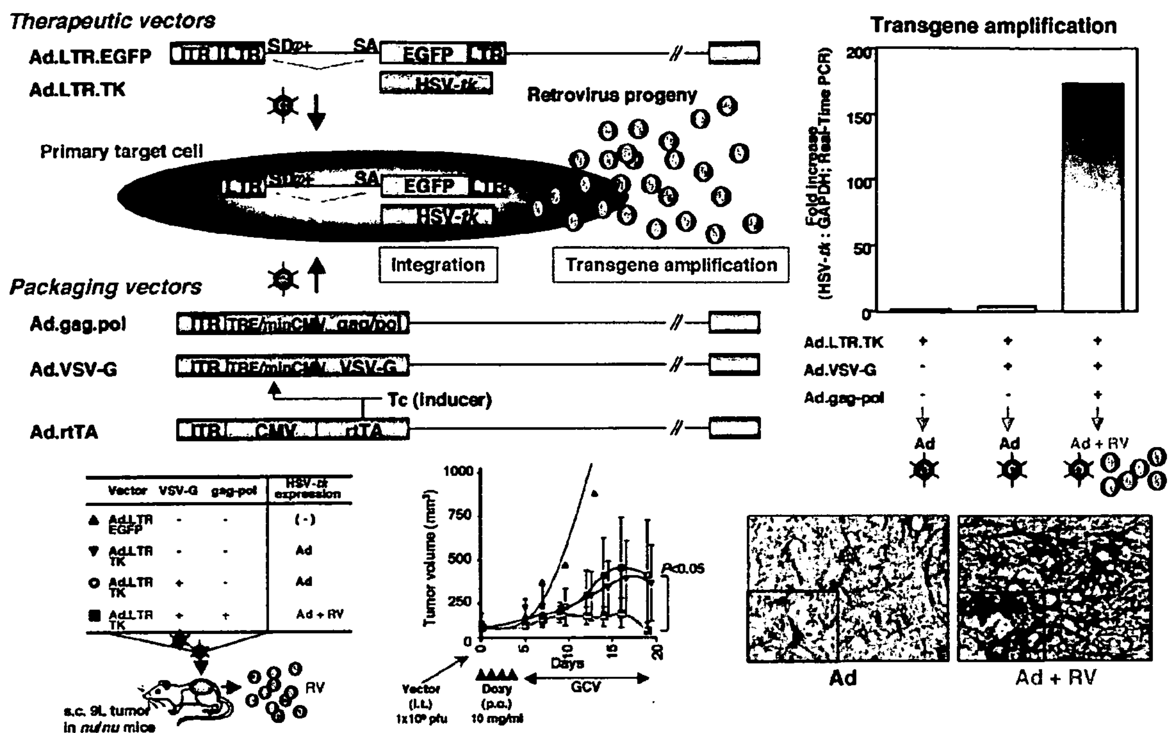

Figure 1. Two adeno-retroviral hybrid vectors containing a retroviral vector genome (Ad.LTR.EGFP or Ad.LTR.TK) were constructed. Co-transduction of rat 9L glioma cells with an adeno-retroviral hybrid vector together with vectors expressing retrovirus packaging proteins (Ad.gag.pol, Ad.VSV-G) as well as an inducer (Ad.rtTA) increased the transduction efficiency. Injection of established subcutaneous 9L tumors on athymic mice with a combination of AVC2.GCTK and packaging vectors followed by GCV treatment resulted in complete regression by 50% of the tumors at day 22, while no tumor regression was observed in control animals. Furthermore, the relative copy number of the HSV-tk gene in tumors treated with the adeno-retroviral vectors was significantly higher than in control tumors. In situ hybridization analysis also suggested dispersion of the HSV-tk product across a wider area of the tumor than in control tumors, which indicates the spread of the in situ-generated retroviruses.

from the efficient gene transfer characteristics of adenoviral vectors as well as the stable and long-term gene expression that is typical of retroviral vectors. We have shown that direct transduction of primary target tumor cells with hybrid adeno-retroviral vectors results in their transient production of recombinant retrovirus particles that then subsequently transduce neighboring tumor cells (3). Moreover, when we transduced established subcutaneous 9L tumors on athymic mice *in situ* with adenovirus vectors that express transcomplementing genes encoding retroviral proteins and retroviral vector RNAs, upon GCV treatment, 50% of the tumors showed complete regression at day 22, while no tumor regression was observed in control animals (Figure 1). This strategy can now be developed further by using cells with tumor-tracking properties as the vector-producing cells, thereby targeting the therapeutic gene to the tumor cells *in vivo*.

3. MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSCs) AS A PLATFORM FOR VECTOR PRODUCTION *IN SITU*

We propose here an improved *in situ* vector production strategy where cells bearing tumor-tracking properties efficiently produce retrovirus- or other virus-based progeny vectors (Figure 2). Candidate tumor-tracking vector-producing cells are adult stem cells. In particular, the fibroblast-like plastic-adherent cells isolated from bone marrow and other sources that are now widely known as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) (4), may be useful as they have been shown to have tumor-seeking properties (5). While the mechanism that induces MSCs to preferentially engraft themselves in tumors remains poorly undertsood, this phenomenon may be mediated by the cytokines released by the tumor or inflammatory tissue. These include hepatocyte growth factor (HGF) (6), vascular endothelial cell growth factor (VEGF) (7), transforming growth factor (TGF) (7), fibroblast growth factor (FGF) (6), platelet-derived growth factor (PDGF) (8), monocyte chemoattractant protein-1 (MCP-1) (9), and IL-8 (9). Moreover, chemokine C-X-C motif receptor 4 (CXCR4), which is present on the surface of an MSC subset, is known to mediate not only the specific migration of MSCs to bone marrow (10), it also governs the migration and homing of a variety of cell types in the developing brain, including neuronal and glial precursors. The only known chemokine that binds with CXCR4 is stromal-cell derived factor-1 (SDF-1). While CXCR4 itself is a major chemokinereceptor on glioma cells and promotes their

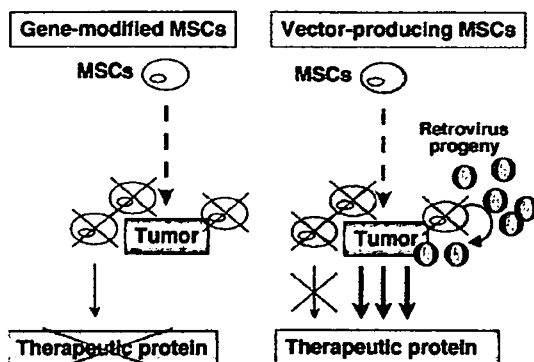

Figure 2. Left panel; Gene-modified MSCs. Although gene-modified MSCs have tumor-seeking properties, the local expression of the therapeutic protein is dependent on the continuing presence of the MSCs. Right panel; Vector-producing MSCs. The retrovirus progeny produced by the MSCs can transduce tumor cells *in situ*, which extends the expression of the therapeutic protein, even when the MSCs die off.

survival (11), high-grade gliomas have recently been found to secrete significant levels of SDF-1 (12). SDF-1 alpha stimulates human glioblastoma cell growth by activating both extracellular signal-regulated kinases 1/2 and Akt. Therefore, CXCR4 expression by MSCs may help them to home to gliomas. When MSCs are infused in mice, they are rapidly and efficiently arrested in the microvasculature (13). Furthermore, these cells are not immunogenic and escape recognition by alloreactive T cells and natural killer cells (14). It also appears that the engraftment of MSCs into the tumor helps them to maintain their stem cell properties *in vivo*. Thus, it appears that MSCs may efficiently engraft human gliomas after intravascular or local delivery and can be used as *in situ* therapeutic vector producers.

Other candidate tumor-tracking cells that may be used as vector-producing cells include endothelial progenitor cells (EPCs). EPCs have been isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are believed to include a hematopoietic stem cell population, and have been shown to incorporate into neovascularization foci (15). Moreover, VEGF promotes adult vasculogenesis by enhancing EPC recruitment and vessel formation at sites of tumor neovascularization (16). Magnetic resonance imaging (MRI) of magnetically labeled endothelial progenitor cells also demonstrated that they traffick to sites of tumor angiogenesis (17).

4. GENETIC MANIPULATION OF MSCs TO FACILITATE THEIR PRODUCTION OF PROGENY VECTORS

MSCs exhibit senescence-associated growth arrest and phenotypic changes during long-term *in vitro* culture. However, overexpression of human telomerase reverse transcriptase (hTERT) in MSCs reconstitutes their telomerase activity and extends their life span (18). Telomerization of MSCs by hTERT overexpression also maintains the stem cell phenotype of MSCs and thus may be useful for generating the numbers of stable MSCs needed for cell differentiation studies and tissue engineering protocols.

To produce therapeutic vectors, the MSCs must be efficiently transduced with viral components. Virus-based transduction techniques have been shown to achieve high gene transduction and transgene expression in many cellular models, and attempts have been made to transduce MSCs with various virus-based vectors such as oncogenic retrovirus- or lentivirus-based vectors. However, the use of integrating viral vectors has several disadvantages, particularly with regard to their safety risks. Many non-viral methods also have limited utility as they are rather inefficient with most primary cells. However, nucleofection, which is a non-viral electroporation-based gene transfer technique, has been shown to be an efficient non-viral transfection technique for MSCs, which then may be used as cellular vehicles for the delivery of biological agents (19). Thus, the Nucleofector technology may be promising as an alternative tool for efficiently transfecting MSCs so that they produce progeny virus.

5. FUTURE DIRECTIONS

Here we propose that current suicide cancer gene therapy strategies may be improved by using vector-producing tumor-tracking MSCs. This strategy is likely to generate *in situ* the vector numbers needed for the killing of solid tumors. We also showed that it may be feasible to produce large-scale preparations of vector-producing cells by transient transduction of MSCs by hybrid adenovirus-based vector infection. It has been shown that the hybrid adenovirus-based vectors that express retroviral proteins can efficiently transduce cells, which then produce progeny vectors (3). However, an impediment for this aim is that MSCs lack the Coxsackie adenovirus receptor (CAR) (20). To overcome this problem, it may be necessary to use a chimeric Ad35 fiber-containing Ad5 vector (21) or a fiber-modified Ad5 vector bearing an RGD-motif peptide in the HI loop of the fiber knob domain (22). Alternatively, it may be possible to use an adaptor molecule that bridges the gap between the viruses and MSCs. Supporting the latter possibility is that we have previously developed a CAR-SCF fusion protein that improves the transduction efficiency of the adenovirus vector with c-kit positive cells (23). Similar CAR-ligand adaptor molecules may be useful for enhancing MSC transduction with the adenovirus vector.

To improve the tumor-targeting properties of the vector-producing cell, how MSCs naturally seek out tumors should be investigated in more detail. In addition, the localization, stability, and vector-producing capacity of gene-manipulated MSCs should be adequately analyzed *in vivo*. Tracking the localization of the MSCs may also help diagnose the recurrence of the disease. Such tracking may be performed by using a molecular imaging technique with MRI. To this end, it has been shown that MSCs labeled with fluorophore particles (IFPs) provide MRI contrast *in vivo* (24). Thus, this type of technology would enable us to

closely study MSC retention, engraftment, and migration in the clinic.

Although previous studies have illuminated the exciting possibilities of suicide cancer gene therapy, in most cases the therapies that were used delivered rather limited clinical benefits. For the sake of safety as well as improving the therapeutic effect of suicide cancer gene therapy, it is important that the suicide gene-expressing vector is accurately delivered to the tumor. This may be achieved by using MSCs to initiate virus production near tumor cells *in situ*. These viruses then transduce the tumor cells, which themselves produce virus progeny, thereby amplifying the transgene expression of the tumor. While the therapeutic benefit of this strategy remains to be tested in various orthotopic or metastatic tumor models, it may be promising for detecting and eradicating evasive tumors *in vivo*.

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Abbreviations: MSCs: mesenchymal stromal cells; GCV: ganciclovir; EPCs endothelial progenitor cells

Key Words: Cancer gene therapy, Multipotent mesenchymal stromal cells, Vector, Review

Send orrespondence to: Takashi Okada M.D., Ph.D., Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551, Japan, Tel : 81-42-346-1720, Fax: 81-42-346-1750, E-mail: t-okada@ncnp.go.jp

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Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy

Takashi Okada, Keiya Ozawa
Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Japan

Abstract

Suicide cancer gene therapy with retroviral vector-producing cells was in the way of an adjuvant to the surgical resection of recurrent glioblastoma, although any benefit appeared to be marginal. Further evaluation of the cancer gene therapy strategy with the vector-producing cells must incorporate improved delivery of vectors and transgenes to the tumor cells. We have previously demonstrated the ability of vector-producing tumor cells engineered by the adenovirus-retrovirus hybrid vector to destroy satellite tumor cells, although therapeutic efficacy for aggressive tumor has to be further evaluated by the systemic delivery of the vector-producing cells. Multipotent mesenchymal stromal cells (MSCs) appear to be effective delivery vehicle to seek out tumor cells *in vivo* and transport cancer-killing gene or immune products with minimal rejection reaction by the host. Therefore, MSCs-mediated tumor transduction with progeny vector production to improve suicide gene therapy might be feasible, if MSCs are capable of producing the recombinant viruses. Although therapeutic benefit in the various orthotopic or metastatic tumor models has to be further validated, this transduction strategy would realize systemic administration of the therapeutic vehicles to detect and eradicate evasive tumors *in vivo*.
(Gene Therapy 2007; p106-111, 2007)

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Takashi Okada
Contact address : Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi, 329-0498, Japan
E-mail: tokada@jichi.ac.jp

Suicide cancer gene therapy with vector producing cells

Antiglioma gene therapy with cells secreting a retroviral vector expressing the herpes simplex thymidine kinase (HSV-*tk*) transgene has been developed (1). This treatment can generate a significant local antitumor effect mediated by tumoricidal metabolites generated by HSV-*tk* activation of the prodrug ganciclovir (GCV). A factor that may enhance the therapeutic value of this technique is the bystander effect, where transduced tumor cells may communicate the apoptosis signal to neighboring cells. However, clinical benefit of this cancer gene therapy system was limited due to the poor efficiency of gene transfer (2). To improve the therapeutic potential of this system, it is necessary to enhance the efficiency of the therapeutic gene delivery *in vivo* as well as to increase the stability of the vector-producing cells.

We have previously described a hybrid vector system that uses the adenoviral vectors to deliver the retroviral vector and packaging proteins into cells (3). The system benefits from the efficient gene transfer characteristics of adenoviral vectors as well as the stable and long-term gene expression that is

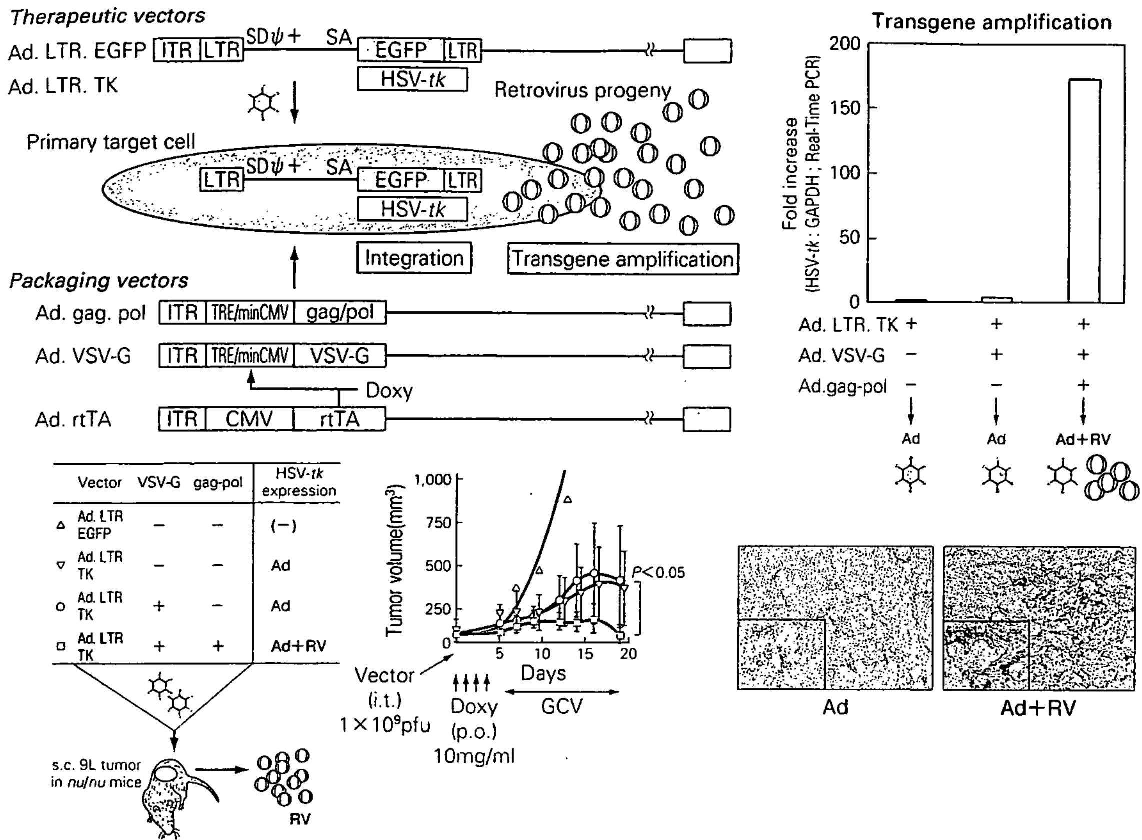

Figure 1 The adeno-retroviral hybrid vector containing the retroviral vector genome was constructed (Ad.LTR.EGFP or Ad.LTR.TK). Co-transduction of rat 9L glioma cells with adeno-retroviral hybrid vector together with vectors expressing packaging proteins of retroviruses (Ad.gag.pol, Ad.VSV-G) as well as an inducer (Ad.rtTA) increased the transduction efficiency. Injection of established subcutaneous 9L tumors on athymic mice with a combination of Ad.LTR.TK and packaging vectors followed by GCV treatment resulted in complete tumor regression in 50% of tumors at day 22, while no tumor regression was observed in control animals. Furthermore, the relative copy number of the HSV-*tk* gene in tumors treated with the adeno-retroviral vectors was significantly higher than that in control tumors. *In situ* hybridization suggested dispersion of the HSV-*tk* product across a wider area of tumor than in control tumors, which indicates the spread of the *in situ* generated retroviruses.

typical of the retroviral vectors. The initial co-transduction of primary target tumor cells with the adeno-retroviral hybrid vectors results in the transient production of recombinant retrovirus particles that then subsequently transduce neighboring tumor cells. Adenovirus vectors expressing transcomplementing genes for retroviral proteins and retroviral vector RNAs have been successfully used for the *in situ* transduction of tumors with enhanced therapeutic effects (Figure 1). A single-step transduction of glioma cells with trans-complementing hybrid adeno-retroviral vectors effectively turned these cells into retrovirus vector producing cells, which in turn facilitates the transduction of adjacent cells. Taking this strategy to the next phase, application of cells with tumor-tracking properties as the vector producing cells would be promising for targeted spatial distribution of the therapeutic gene *in vivo*.

Multipotent mesenchymal stromal cells (MSCs) as a platform for vector production *in situ*

We propose here an improved *in situ* vector production strategy that efficiently produces the progeny vectors based on retrovirus or

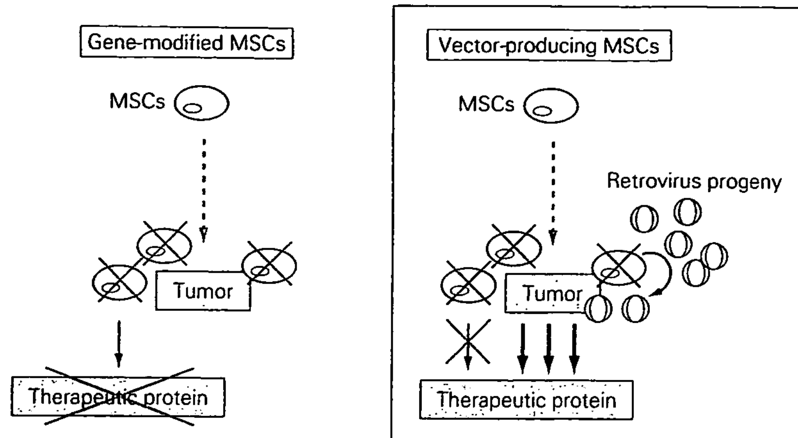

Figure 2
Left panel; Gene-modified MSCs
Although gene-modified MSCs have tumor-seeking property, local expression of the therapeutic protein is up to the presence of the MSCs.
Right panel; Vector-producing MSCs
Retrovirus progeny produced by the MSCs can transduce tumor cells *in situ* to amplify the therapeutic genes with extended expression of the therapeutic protein, even when MSCs fade away.

others [Figure 2]. To render the tumor-tracking properties for the vector-producing cells, important types of adult stem cells can be considered. The fibroblast-like plastic-adherent cells isolated from bone marrow and other sources have come to be widely known as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) (4). The tumor-seeking property of MSCs has been demonstrated, although mechanisms are poorly understood (5). This engraftment may be mediated by the cytokines released from the tumor or inflammatory tissue, such as hepatocyte growth factor (HGF) (6), vascular endothelial cell growth factors (VEGF) (7), transforming growth factor (TGF) (6), fibroblast growth factor (FGF) (6), platelet-derived growth factors (PDGF) (8), monocyte chemoattractant protein-1 (MCP-1) (9), and IL-8 (9). Chemokine C-X-C motif receptor 4 (CXCR4), although presents at the surface of a subset of MSCs, is important for mediating specific migration of MSCs to bone marrow (10). CXCR4 is known to govern cellular migration and homing in a variety of cell types, including neuronal and glial precursors in the developing brain. The only known chemokine that binds with CXCR4 is stromal-cell derived factor-1 (SDF-1). While CXCR4 itself is a major chemokine receptor on glioma cells and mediates their survival (11) , high-grade gliomas have recently been found to secrete significant levels of SDF-1 (12). SDF-1 alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Therefore, CXCR4 would render MSCs with homing activity to the glioma with growth stimulation. The pattern of organ distribution suggested that infused cells were efficiently arrested in microvasculature during first-pass (13). Furthermore, these cells are not immunogenic and escape recognition by alloreactive T cells and natural killer cells (14). If MSCs efficiently integrate into human gliomas after intravascular or local delivery, MSCs can be exploited to take therapeutic advantage of vector production *in situ*. Engraftment

of MSCs into the tumor is also important to maintain their stem cell properties *in vivo*.

Other candidates of cells with tumor-tracking properties as the vector producing cells are including endothelial progenitor cells (EPCs). EPCs were isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are considered to include a hematopoietic stem cell population, and were shown to be incorporated into foci of neovascularization (15). VEGF promotes adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization (16). Magnetic resonance imaging (MRI) of magnetically labeled endothelial progenitor cells demonstrated trafficking to sites of tumor angiogenesis (17).

Genetic manipulation of MSCs to produce progeny vectors

MSCs exhibit senescence-associated growth arrest and phenotypic changes during long-term *in vitro* culture. Overexpression of human telomerase reverse transcriptase (hTERT) in MSCs reconstitutes telomerase activity and extends life span of the cell (18). Telomerization of MSCs by hTERT overexpression maintains the stem cell phenotype of MSCs and it may be a useful tool for obtaining enough number of cells with a stable phenotype for cell differentiation studies and tissue engineering protocols.

Comprehensive assessment of the capability for the vector production with MSCs should be evaluated by the transduction of the MSCs with the viral components. Viral-based transduction techniques are efficient systems to deliver DNA into stem cells because they show high gene transduction and transgene expression in many cellular models. Various virus-based vectors, such as oncogenic retrovirus vectors or lentivirus-based vectors have been attempted to transduce MSCs. However, the use of integrating viral vectors has several disadvantages mainly involving safety risks. However, non-viral methods are rather inefficient for most primary cells. Nucleofection is an efficient non-viral transfection technique for MSCs, which then may be used as cellular vehicles for the delivery of biological agents (19). The Nucleofector technology, a non-viral electroporation-based gene transfer technique, seems to be promising as an efficient tool for transfecting MSC to produce progeny virus.

Future direction

Here we have proposed a feasibility of the vector-producing tumor-tracking cells to improve suicide cancer gene therapy. This strategy with MSCs would produce sufficient levels of vectors *in situ* for the killing of solid tumors. Furthermore, transient transduction of the MSCs with the adenovirus-based hybrid vector infection in a large-scale preparation of the VP-MSCs should realize the clinical investigation. The adenovirus-based hybrid vector with vectors expressing retroviral proteins can efficiently transduce cells to produce progeny vectors (3). In this context, the absence of the Coxsackie adenovirus receptor (CAR) on the MSCs seems to be a bottleneck (20). To overcome this issue, a chimeric Ad5 vector containing an Ad35 fiber (21) or a fiber-modified Ad5 vector containing an RGD-motif peptide in the HI loop of the fiber knob domain (22) was attempted to increase transduction efficiency. Alternatively, an adaptor molecule to bridge the gap between the viruses and MSCs may be applicable. We have previously developed a CAR-SCF fusion protein to improve transduction efficiency of the c-kit positive cells with the adenovirus vector (23). Similar CAR-ligand adaptor molecule would be of use to enhance transduction of the MSCs with the adenovirus vector.

For the improved tumor-targeting properties, mechanistic insight of the tumor-seeking