1.**NF-κB activity regulates mesenchymal stem cell accumulation at tumor sites**

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Conflict of interest
The authors have declared that no conflict of interest exists.

Running title
Mechanisms of MSC accumulation at tumor sites

Keywords
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Abstract

Mesenchymal stem cells (MSCs) accumulate at tumor sites when injected into tumor-bearing mice, perhaps offering cellular vectors for cancer-targeted gene therapy. However, the molecular mechanisms involved in MSC targeting to tumors are presently little understood. We focused on MSC-endothelial cell (EC) adhesion following tumor necrosis factor-α (TNF-α) stimulation in an attempt to elucidate these mechanisms. Interestingly, stimulation of MSCs with TNF-α enhanced the adhesion of MSCs to ECs in vitro. This adhesion was partially inhibited by blocking antibodies against vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4). It is well known that TNF-α induces VCAM-1 expression via the NF-κB signaling pathway. Parthenolide (PTL) has an anti-inflammatory activity and suppressed NF-κB activity by inhibition of IκBα phosphorylation after TNF-α stimulation, and strongly inhibited TNF-α-induced VCAM-1 expression on MSCs. In vivo imaging using luciferase-expressing MSCs revealed that the bioluminescent signal gradually increased at tumor sites in mice injected with untreated MSCs. In contrast, we observed very weak signals at tumor sites in mice injected with PTL-treated MSCs. Our results suggest that NF-κB activity regulates MSC accumulation at tumors, by inducing VCAM-1 and thereby its interaction with tumor vessel ECs. These findings have implications for the ongoing development of efficient MSC-based gene therapies for cancer treatment.
Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells with high proliferative potency and have the ability to differentiate into multiple lineages. They are detected in several adult and fetal tissues, including bone marrow, adipose tissue, and umbilical cord blood. MSCs have generated a great deal of interest in their potential use in regenerative medicine due to their ability to migrate to damaged tissues and to produce cytokines. Furthermore, MSCs can be easily genetically modified with viral vectors to be employed as novel cellular vehicles in gene therapy protocols. MSCs are also utilized to treat severe acute graft-versus-host disease, because they accumulate at inflammatory lesions and have immunomodulatory activity.

Interestingly, recent studies indicated that MSCs also have the ability to home to tumors. Therefore, they can be used as cellular vehicles for cancer-targeted gene therapy. Intravenous injection of engineered MSCs expressing interferon-β was reported to inhibit the growth of melanoma pulmonary metastasis (1) and breast cancer (2) in mice, and also prolonged the survival of mice with glioma xenografts (3). Furthermore, interleukin (IL)-12, which improves immune surveillance against cancer cells (4), and chemokine CX3CL1 (fractalkine), which is able to activate T cells and NK cells (5), were used as therapeutic molecules. We have also demonstrated that retrovirus vector-producing MSCs also effectively inhibit tumor growth (6). In this context, treatment has been developed using retroviral vectors expressing the thymidine kinase of Herpes Simplex Virus combined with the pro-drug ganciclovir.

The ability of MSCs to specifically localize to multiple tumors makes them extremely
attractive for targeted cancer therapy. The most likely cause of preferential migration was considered to be the release of chemotactic gradients from tumor tissues. MSCs have a variety of chemokine and cytokine receptors, and respond functionally to ligands in vitro. Tumors are known to produce a large amount of chemokines and cytokines, which could serve as ligands for the receptors on MSCs (7). Therefore, the mechanism of MSC accumulation at the site of tumors seems to be based on their migratory ability. Nevertheless, although various growth factors and chemokines such as platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and stromal cell-derived factor-1α (SDF-1α) may be involved, the detailed molecular mechanisms of MSC accumulation at tumors are poorly understood.

In the present study, we focused on MSC-endothelial cell (EC) adhesion following TNF-α stimulation in an attempt to elucidate the mechanism of MSC accumulation at tumors.
Materials and Methods

Cell culture

Bone marrow-derived human MSCs (Lonza Walkersville, Inc., Walkersville, MS) were cultured in mesenPRO RS medium (Invitrogen, Grand Island, NY). HEK293-derived AD-293 cells (Stratagene, La Jolla, CA), human embryonic fibroblasts WI-38 (FBs; RIKEN BRC, Ibaraki, Japan), human colon adenocarcinoma cell lines SW480 (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan) and SW480/RFP that was generated by transduction of SW480 with red fluorescent protein-expressing retrovirus vectors (RV-RFP), were grown in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 Units/ml penicillin, and 100 µg/ml streptomycin (P/S). Human endothelial progenitor cells (ECs; ApproCell Inc., Hayward, CA) were cultured in EPC grown medium (ApproCell Inc.). Human colon adenocarcinoma cell lines Colo205 (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University) and Colo205/RFP that was generated by transduction with RV-RFP, were grown in RPMI medium (Invitrogen) supplemented with FBS and P/S. All cultures were kept in an incubator at 37°C and 5% CO₂.

Adenoviral vectors

Adenoviral vectors expressing a green fluorescent protein (GFP) were constructed by an improved in vitro ligation method (8, 9). The shuttle plasmid pHMCA5-GFP contains a CA promoter (a β-actin promoter/CMV enhancer with a β-actin intron), GFP gene, and
a bovine growth hormone (BGH) polyadenylation signal, all of which are flanked by
I-CeuI and PI-SceI restriction sites. I-CeuI/PI-SceI-digested pHMCA5-GFP was ligated
with I-CeuI/PI-SceI-digested pAdHM4, resulting in pAdHM4-CAGFP. pAdHM41-K7-CAGFP was constructed by ligation of I-CeuI/PI-SceI-digested
pHMCA5-GFP with I-CeuI/PI-SceI-digested pAdHM41-K7 (10). Viruses (Ad5-GFP
and AdK7-GFP) were generated by transfection of PacI-digested pAdHM4-CAGFP and
pAdHM41-K7-CAGFP, respectively, into AD-293 cells with SuperFect (Qiagen,
Valencia, CA) according to the manufacturer’s instructions. Each virus was purified by
CsCl2 step gradient ultra-centrifugation followed by CsCl2 linear gradient
ultra-centrifugation. Virus particles and biological titers of each vector preparation were
determined as described by Mittereder et al. (11). We also created Ad vectors expressing
luciferase (Luc) using the shuttle plasmid pHMCA5-Luc, which contains the Luc gene
derived from pELuc-test (Toyobo Co. Ltd., Osaka, Japan). MSCs and FBs were seeded
in culture plates or flasks at a density of 1x10^4 cells/cm^2, and the next day the cells were
treated with each adenovirus vector for 1.5 h. The medium containing the vectors was
removed and replaced with fresh medium.

Animal models

All animal experiments were approved by the Jichi Medical University ethics committee
and performed in accordance with the National Institute of Health Guide for the Care
and Use of Laboratory Animals. To create tumor-bearing mice, SW480/RFP cells (3 x
10^6) were subcutaneously inoculated into 4- to 6-week-old male Balb/c nu/nu mice
(Clea Japan Inc, Tokyo, Japan). The mice were used for experiments 7 days after inoculation.

**Immunohistchemistry**

Cultured MSCs and FBs were transduced with AdK7-GFP at a concentration of 3,000 virus particles/cell (vp/cell). Two days after transduction, cells were injected into the left ventricular cavities (1 x 10^6, day 0) of tumor-bearing mice. Mice were sacrificed on day 4, and 7 μm serial cryosections from frozen tissues were processed. Immunohistochemistry was performed with FITC-conjugated anti-GFP antibody (ab6662; Abcam Inc., Cambridge, MA, USA) on tumor cryosections to detect MSCs or FBs. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Images were obtained with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). SW480/RFP cells (3 x 10^6) were subcutaneously inoculated into 4- to 6-week-old male Balb/c nu/nu mice. Mice were sacrificed on day 11, serial sections from tumor tissues were processed. Immunohistochemistry was performed with anti-mouse CD34 monoclonal antibody (MEC14.7; GeneTex Inc., San Antonio, TX, USA) on tumor section to detect tumor blood vessels. Histofine Simple Stain Mouse MAX PO (Nichirei Biosciences, Inc., Tokyo, Japan) was used as a horseradish peroxidase-conjugated secondary antibody, and DAB solution was used for brown color development. Sections were then counterstained with hematoxylin Hematoxilin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Images were obtained with a fluorescence microscope (BZ-9000).
In vivo imaging of homing ability to tumors

Cultured MSCs and FBs were transduced with AdK7-Luc at a concentration of 3,000 vp/cell and 680 vp/cell, respectively. Two days after transduction, cells were injected into the left ventricular cavities (1 x 10^6, day 0) of tumor-bearing mice, and then optical bioluminescence imaging was performed to periodically trace the cells using an in vivo imaging system (IVIS; Xenogen, Hopkinton, MA, USA). To detect bioluminescence from MSCs or FBs, the reporter substrate D-luciferin (Ieda Chemical Co., Ltd., Tokyo, Japan) was injected into the mouse peritoneum (75 mg/kg body weight) for scanning. The luminescent intensity at tumor sites was analyzed using Living Image software (Xenogen, Alameda, CA).

In vitro migration assays

Cultured MSCs or FBs were serum-starved for 12 h. One hundred microliters of tumor conditioning medium, or serum-free medium supplemented with PDGF-BB (10 ng/ml), HGF (30 ng/ml), FGF-β (20 ng/ml), SDF-1α (150 ng/ml), VEGF-A (25 ng/ml), or monocyte chemoattractant protein-1 (MCP-1; 100 ng/ml) was added to the lower wells of migration chambers (8 μm pore size; Neuro Probe, Inc., Gaithersburg, MD, USA); MSCs or FBs (4 x 10^4) were added to the upper wells. All recombinant proteins were purchased from R&D systems Inc. (Minneapolis, MN, USA). Medium alone (DMEM/F-12) was used as a negative control and treatment with 30% FBS was the positive control. After incubation for 24 h at 37°C, cells were labeled with CyQUANT
NF dye, and cells attached to the lower surface of the filters were detached with trypsin.

Fluorescent intensity was measured using a fluoroscan, and the number of adherent cells
was quantified using a standard curve constructed by a known number of cells.

Flow cytometric analysis of adhesion molecules
Cultured MSCs, FBs or ECs were stimulated with TNF-α and harvested by
trypsinization. Cell aliquots were incubated with fluorescein isothiocyanate
(FITC)-conjugated monoclonal antibodies (BD) against VCAM-1, CD49d, CD29
(Integrin-β1), and analyzed by flow cytometry (FACScan; BD Biosciences, San Jose,
CA). For each analysis, an aliquot of cells was also stained with isotype control
IgG-conjugated to FITC as a negative control.

Assay for TNF-α produced in tumor-bearing mice
SW480/RFP (3 x 10^6) cells were subcutaneously inoculated into nude mice. Seven days
after inoculation, mice were anaesthetized with an overdose of isoflurane inhalation.
The blood was collected and allowed to coagulate overnight on ice. After centrifugation
of the samples (2,000 x g, 30 min, 4°C), the serum was removed and stored at −70°C.
Tumor, spleen and liver tissues were homogenized in 1.5 ml of α-MEM medium using a
tissue homogenizer. The homogenates were then centrifuged (2,000 x g, 30 min, 4°C)
and the supernatant was removed and re-centrifuged (14,000 x g, 30 min at 4°C). Serum
and supernatants from tissue homogenates were kept at −70°C until use. TNF-α was
assayed using a commercially available ELISA kit (mouse TNFα Instant ELISA;
**In vitro adhesion assays**

For adhesion assays, ECs (at 4 passages) were cultured to confluence on fibronectin-coated 96-well plates (20 ng/ml; Sigma Aldrich, Inc., St. Louis, MO, USA) and treated with TNF-\(\alpha\) (10 ng/ml) for 12 h prior to assaying. MSCs and FBs were treated with TNF-\(\alpha\) (10 ng/ml) 12 h before the adhesion assays, and incubated with isotype control IgG or anti-VCAM-1 or VLA-4 (10 \(\mu\)g/ml) mAbs for 1 h. Cells were labeled with CyQUANT NF dye, and 1 \(\times\) 10^4 cells were seeded onto ECs. After 30 min incubation at 37°C, wells were washed thoroughly three times with PBS to remove non-adherent cells. Fluorescent intensity was measured using a fluoroscan, and the number of adherent cells was quantified using a standard curve constructed by a known number of cells. In some experiments, MSCs and FBs were pretreated for adhesion studies with one of the following substances: TNF-\(\alpha\) (10 ng/ml), anti-VCAM-1 antibody (mouse monoclonal anti-rat, clone 5F10, 10 \(\mu\)g/ml, Eurogentec), or anti-VLA-4 antibody (mouse monoclonal anti-rat, clone 1A29, 10 \(\mu\)g/ml, Research Diagnostics).

**Parthenolide treatment of MSCs**

Parthenolide (PTL; Biomol, Plymouth Meeting, PA, USA) was reconstituted in dimethyl sulfoxide (DMSO; Sigma Aldrich, Inc.) to a stock concentration of 0.4 M and subsequently diluted in PBS. MSCs were treated with PTL (5 \(\mu\)M) for 6 h before experiments. To assess the effect of PTL treatment for transgene expression, cells were
re-seeded into 96-well plates, and luciferase assays were performed using luciferase-expressing MSCs. Cell viability after PTL treatment was also examined with Cell Proliferation Kit II (XTT; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Western blotting

Western blot analysis was performed to measure the NF-κB pathways. Next, MSCs were pretreated with PTL or vehicle (DMSO) for 6 hours, then cultured with TNF-α (10 ng/ml) for 3 min. Cells were lysed in RIPA buffer containing protease inhibitor (Pierce Biotechnology, Rockford, IL, USA). Protein extracts were electrophoresed on a 4-12% Bis-Tris gel (Invitrogen), and transferred to PVDF membranes. Membranes were incubated in PVDF blocking reagent (TOYOBO), and then incubated with primary antibodies against the following proteins: IκB-α, phospho- IκB-α (Ser32), NF-κB p65, phospho-NF-κB p65 (Ser536), and α-tublin (Cell Signaling Technology, Beverly, MA), followed by incubation with HRP-conjugated goat anti-rabbit IgG or –mouse IgG1 secondary antibody, and detected using a western blotting detection system (GE Healthcare, Buckinghamshire, UK).

Immunocytochemistry

To visualize p65 nuclear translocation, MSCs were pretreated with PTL or vehicle (DMSO) for 6 hours, then cultured with TNF-α (10 ng/ml) for 20 min. Cells were fixed with 4% formaline and permeabilized with Triron-X 100. After washing with PBS, slides
were incubated with rabbit anti-p65 antibody (Cell Signaling Technology), followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody. The actin cytoskeleton was stained with Alexa Fluor 546-conjugated phalloidin (Invitrogen); nuclei were stained with DRAQ-5 dye (Invitrogen). Cells were examined using Keyence BZ-9000.
Results

In vivo imaging of MSC accumulation in tumors

We used bone marrow-derived human MSCs, which expressed characteristic phenotypic markers for MSCs and differentiated into adipocyte, osteocyte, and chondrocyte under specific culture conditions (Supplemental Fig. 1). Then, fiber-modified adenovirus vectors (AdK7) were used for efficient transduction of MSCs and fibroblasts in this study. When the cells were transduced with GFP-expressing Ad.K7 vectors at a density of 3,000 vp/cell, transduction efficiency was almost 100% (Supplemental Fig. 2A and B). The bioluminescent intensity of MSCs transduced with luciferase-expressing Ad vectors at 3,000 vp/cell was equal to that of FBs transduced at 680 vp/cell (Supplemental Fig. 2C). Mice injected with GFP-expressing MSCs or FBs were sacrificed 4 days after injection for immunohistochemical analysis. MSCs identified with anti-GFP antibody were detected in the boundaries of tumors and tumor stroma. However, we found no GFP-positive FBs in the tumor tissues (Fig. 1A). We also used bioluminescence imaging to quantitatively investigate the tumor tropism of MSCs. We injected luciferase-expressing MSCs or FBs into mice through the left ventricular cavity, and then performed optical bioluminescence imaging to periodically trace the cells using IVIS. In mice injected with luciferase-expressing MSCs, optical bioluminescence at tumor sites became pronounced over time (Fig. 1B), and signal intensity gradually increased (Fig. 1D). In contrast, we observed no signal at the tumor sites in mice injected with luciferase-expressing FBs (Fig. 1C and D).

In vitro migration assays
We analyzed the effects of several growth factors (specifically PDGF-BB, HGF, and vascular endothelial growth factor (VEGF)), chemokines (specifically MCP-1 and SDF-1α), and SW480 culture-conditioned medium on MSC and FB migration. These factors are commonly expressed in tumor tissues, and are thought to be potential mediators of MSC tropism. We also used serum-free medium as a negative control, and medium containing 30% FBS as a positive control. Migration was quantified by direct labeling and counting of cells by a fluorometer (Fluoroskan Ascent FL; Thermo Labsystems, Helsinki, Finland). Exposure to PDGF, HGF, or conditioned medium from SW480 cells stimulated significant MSC migration, whereas VEGF and SDF-1α had no significant effect compared with serum-free medium (Fig. 2). We compared the migration capacity of MSCs and FBs, the factors that attracted MSCs also induced migration of FBs. Rather, it appears that FBs were more strongly attracted to these factors than MSCs.

**In vitro adhesion assays**

The tumors generated in mice in this study strongly induced tumor stroma with defined blood vessels, and MSCs specifically accumulated in this stroma (Fig. 3A). Therefore, we propose a hypothesis as follows: factors, as indicated in Fig. 2, attract both MSCs and FBs to the tumor microenvironment, but importantly, MSCs significantly adhere to ECs compared to FBs. Therefore, only MSCs migrate and accumulate at tumor sites via blood vessels in tumor stroma. We speculated that inflammatory cytokines (specifically TNF-α) are required for induction of adhesion molecule expression. First, we measured
TNF-α levels in tumor tissues by ELISA. The TNF-α level is significantly higher in tumor tissues compared to liver and spleen (Fig. 3B). Similar results were also observed in another experiments using Colo205 tumor cells (Supplemental Fig. 3). Then, we assessed the expression of adhesion molecules on ECs, MSCs, and FBs by FACS analysis. After TNF-α stimulation, ECs and MSCs significantly expressed adhesion molecules including VCAM-1 and VLA-4, compared to FBs (Fig. 3C). We also examined the in vitro adhesion of MSCs to ECs. MSCs effectively adhered to ECs compared to FBs (Fig. 3D). Furthermore, this adhesion was partially inhibited by blocking antibodies against VCAM-1 and VLA-4.

Effects of parthenolide on MSC migration and adhesion
We propose a hypothesis that if TNF-α-induced VCAM-1 expression is inhibited, MSC accumulation at tumors is also attenuated. It is well known that TNF-α induces VCAM-1 expression through the NF-κB signaling pathway. We used PTL, a sesquiterpene lactone that occurs naturally in the Feverfew plant. Although PTL has several biological activities, we focused on its suppressive effect on NF-κB activity. At first, there were no differences in migratory capacity towards growth factors or chemokines with or without PTL treatment (Fig. 4A). Next, we assessed the inhibitory effect of PTL on NF-κB activity: MSCs were pretreated for 6 h, and then were stimulated with TNF-α for 3 min. PTL suppressed p65 nuclear translocation through the inhibition of IκBα phosphorylation (Fig. 4B and C) and strongly inhibited the TNF-α-induced VCAM-1 expression on MSCs (Fig. 4D). Consequently, and MSC-EC
adhesion was strongly inhibited by PTL treatment similarly to anti-VCAM-1 blocking antibody (Fig. 4E).

In vivo imaging of PTL-treated MSCs

First, we examined the effect of PTL treatment on transgene expression and cell viability. There were no significant effects on transgene expression and cell viability after PTL treatment (Fig. 5A and B). Next, we performed in vivo imaging using IVIS. We observed definite bioluminescence at tumor sites in the mice injected with untreated MSCs (Fig. 5C), and bioluminescent intensity was gradually increased (Fig. 5E), as indicated earlier (Fig. 1B). In contrast, we could not observe definite accumulation at the tumor sites in mice injected with PTL-treated MSCs (Fig. 5D and E). Similar results were also obtained by experiments using Colo205 tumor-bearing mice (Supplemental Fig. 4).
Discussion

In this study, we demonstrated that MSC accumulation at tumor sites would be related not only to migratory capacity towards growth factors and chemokines, but to MSC-EC adhesion following activation by TNF-α. We further showed that NF-κB activity regulates MSC accumulation at tumor sites through the induction of VCAM-1 expression and the resultant interaction with tumor blood vessel ECs.

It is thought that MSCs are mobilized into action following tissue damage, such as injury or inflammation typically accompanied by the release of inflammatory cytokines from the damaged tissues, leading to the recruitment of MSCs to the target. Tumors have a microenvironment consisting of large numbers of inflammatory cells (12). This microenvironment promotes the recruitment of MSCs via various soluble factors secreted by the tumor and inflammatory cells, including epidermal growth factor (EGF), VEGF-A, FGF, PDGF, SDF-1α, IL-8, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), MCP-1, HGF, transforming growth factor-β1 (TGF-β1), and urokinase-type plasminogen activator (uPA) (13). However, in our experimental settings, although systemically injected MSCs accumulated at the tumors, subcutaneously injected MSCs did not (data not shown). We also compared the migration capacity of MSCs and FBs towards growth factors and chemokines in vitro. Rather, it appears that FBs were more strongly attracted to these factors than MSCs. Our results suggest that the mechanism of MSC accumulation cannot be explained solely by cytokine-mediated migration. Therefore, we need different viewpoints to clarify the mechanism.
The tumors generated in this study strongly induced tumor stroma with large numbers of blood vessels, and MSCs in particular accumulated in the boundaries between the tumors and tumor stroma. Furthermore, MSC accumulation at the site of the tumors was observed only when cells were injected via the left ventricular cavity. Therefore, we focused on MSC-EC adhesion in order to elucidate the mechanisms involved.

It has previously been reported that the interaction of MSCs with the vascular endothelium resembles leukocyte chemotaxis (14). To analyze these interactions, we referred to a model that has been proposed for endothelial cell regulation of leukocyte infiltration in inflammatory tissues. Leukocyte-endothelial adhesion involves dynamic interactions between leukocytes and endothelial cells, and involves multiple steps. These steps must be precisely orchestrated to ensure a rapid response with minimal damage to healthy tissue (15). Interactions between leukocytes and the endothelium are mediated by several families of adhesion molecules, each of which participates in a different phase of the process. The surface expression and activation of these molecules during an inflammatory response is tightly controlled under normal conditions. Inflammatory cytokines including IL-1 and TNF-α involve induction of adhesion molecules. In our experimental settings, although other inflammatory cytokine levels including IL-1 and IL-6 were low (data not shown), significant production of TNF-α was observed. We do not clearly know the source of TNF-α in the tumor at this time and that our in vitro data only suggest that the stroma is the primary source.

As we expected, TNF-α enabled MSCs to adhere to ECs through induction of the expression of adhesion molecules, including VCAM-1 and VLA-4. It is generally
considered that VCAM-1 on activated endothelium interacts with the VLA-4 on the leukocyte in the model of leukocyte-endothelial cell adhesion. At first, we speculated that VLA-4 on MSCs plays the same important role as leukocytes. Whereas both VCAM-1 and VLA-4 on endothelium were efficiently induced by TNF-α stimulation, TNF-α-induced expression of VCAM-1 on MSCs is much stronger than that of VLA-4. Furthermore, MSC-EC adhesion was more effectively inhibited by anti-VCAM-1 antibody as compared to the anti-VLA-4 antibody. Based on these results, although VLA-4 on MSC have also related to the MSC/EC adhesion, we thought that VCAM-1 on MSC has more important implications for this adhesion. Once MSCs circulate in the bloodstream, adhesion to ECs is the first step in accumulation in tumors. TNF-α exerts its biological functions through activating the NF-κB signaling pathway. NF-κB is a major cell survival signal that is anti-apoptotic. MSC accumulation was significantly decreased through parthenolide inhibition of NF-κB activity. Although several studies have shown that MAPK phosphorylation by growth factors are involved in MSC migration (16, 17), PTL did not inhibit MAPK phosphorylation (data not shown). Therefore, at least PTL treatment did not affect in migration ability of MSCs towards growth factors from tumors in this experimental settings. Nevertheless, MSC accumulation was significantly decreased through PTL inhibition of NF-κB activity. We did not show histological evidence in the experiments using PTL. However, we demonstrate that PTL does not inhibit luciferase activity in vitro (and thus does not seem to be toxic), and that therefore the effect observed in vivo should be an effect on recruitment. Although we focused on the function of TNF-α in this study, other
inflammatory cytokines including IL-1β and IFN-γ also have ability to induce VCAM-1 expression in target cells (18), and may be involved in MSC accumulation.

TNF-α is a major inflammatory cytokine that plays important roles in diverse cellular events such as cell survival, proliferation, differentiation, and death. Numerous reports have shown that TNF-α levels in serum are increased in cancer patients (19, 20), and TNF-α is also related closely to the tumor progression including metastasis. For example, TNF-α intensely induces IL-6 and MCP-1 from cancer-associated fibroblasts and normal fibroblastic cells and has indirect influences on generation of prometastatic microenvironment (21). Further, TNF-α is also released in cardiac infarction, during acute coronary syndromes, and in chronic heart failure; MSCs also accumulate at the site of cardiac infarction (22, 23). These results indicated that pro-inflammatory cytokines promote homing of stem cells in the heart and that these cytokines have a positive effect on cardiac regeneration. Therefore, activation with TNF-α is one of the critically important steps for MSC accumulation. Moreover, MSC-based tissue-targeted strategies may be adapted for various inflammatory diseases.

In MSC-based cancer-targeted gene therapies, it is thought that therapeutic efficacy is directly linked with accumulation efficiency of MSCs at tumor sites. Our results suggested that combination use of NF-κB inhibitors, including bortezomib, or TNF-α blocking agents, such as infliximab, reduces the therapeutic efficacy of gene-modified MSCs due to inhibition of the accumulation steps. By contrast, tumor-specific TNF-α-inducing agents would be useful in enhancing therapeutic efficacy, thus further research is required in identifying such agents to more effective therapeutic strategies.
In conclusion, the present study demonstrates that NF-κB activation through TNF-α stimulation and VCAM-1/VLA-4-mediated MSC/EC adhesion may be an important element in MSC accumulation. Although MSCs are useful as cellular vehicles for cancer-targeted gene therapy, past studies have shown that increased MSC accumulation is needed to enhance therapeutic efficacy. Thus, methodology for the enhancement of MSC accumulation should be developed, and our findings suggest a solution.

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References


23. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac
Figure Legends

Figure 1. Tumor homing ability of MSCs in vivo. (A) Subcutaneous tumors were induced by injection of SW480/RFP cells (3 x 10^6) in nude mice (day 0). Cultured MSCs or FBs were transduced with GFP-expressing adenovirus vectors 2 days before injection (day 5), and were injected into the left ventricular cavity (1 x 10^6, day 7). Mice were sacrificed on day 11, and immunohistochemistry was performed with anti-GFP antibody on tumor cryosections to detect MSCs or FBs. Top, fluorescent microscopy view of MSC detection, MSCs (left), RFP-labeled tumor cells (center), nucleic staining with DAPI and merge (right). Bottom, fluorescent microscopy view of FB detection, FBs (left), RFP-labeled tumor cells (center), nucleic staining with DAPI and merge (right). Data shown are from one representative experiment of three performed. Scale bar; 100 µm. S, stroma; T, tumor. (B) Luciferase-expressing MSCs were injected into tumor-bearing mice via the left ventricular cavity (1 x 10^6, day 7). Optical bioluminescence imaging was performed to periodically trace the cells using IVIS. Upper, biodistribution of MSCs as detected by luminescence. Lower, tumor site detected by red fluorescence. Data shown are from one representative experiment of eight performed. (C) Luciferase-expressing FBs were injected into tumor-bearing mice and IVIS imaging was performed as described above. Upper, biodistribution of FBs indicated by luminescence. Lower, tumor site indicated by red fluorescence. Data shown are from one representative experiment of seven performed. (D) Bioluminescent intensity at tumor sites was quantified using analysis software. The data are expressed as means ± SD (n=8 for MSCs and n=7 for FBs). *P<0.05, **P<0.01 compared with
Figure 2. Migratory capacity of MSCs and FBs in response to growth factors, chemokines, and conditioned medium from SW480 cells. MSCs or FBs were serum-starved for 12 h. Cells (4 x 10^4) were added to upper wells of migration chambers. Then, tumor conditioning medium, serum-free medium supplemented with PDGF-BB (10 ng/ml), HGF (30 ng/ml), bFGF (20 ng/ml), SDF-1α (150 ng/ml), VEGF-A (25 ng/ml), or MCP-1 (100 ng/ml) were added to the lower wells. Treatment with medium alone (DMEM/F-12) was used as a negative control and treatment with 30% FBS was used as the positive control. The contents of the upper wells and lower wells were separated by polycarbonate filters (8 µm). The data are expressed as means ± SD (n=8 per cell type). Values are presented as means ± SE. *P<0.05 and **P<0.01 compared with each control.

Figure 3. (A) Sections represent HE staining (upper left), the CD34+ blood vessels/endothelial cells in tumor tissues (upper right), the high power field of view (lower). Data shown are from one representative experiment of three performed. Scale bar; 100 µm. S, stroma; T, tumor. (B) Specimens of tumor, liver, spleen, and blood were collected from control and tumor-bearing mice. TNF-α levels in tissue homogenates and serum were assayed by ELISA. *P<0.05, **P<0.01. (C) MSCs, ECs, and FBs were cultured with TNF-α (10 ng/ml) for 6 hours. Cells were labeled with FITC-conjugated antibodies and analyzed by flow cytometry (filled histogram). Rat isotype antibodies
IgG1 and IgG2a served as respective controls (open histograms). Values represent the percentage of positive cells after TNF-α stimulation, and values in parentheses represent the percentage of positive cells without TNF-α stimulation. (D) ECs were cultured to confluence on fibronectin-coated 96-well plates. Then, MSCs or FBs (1 x 10^4) were added to cultured ECs. MSCs and ECs were pretreated with the following substances: TNF-α (10 ng/ml), anti-VCAM-1, VLA-4 (10 µg/ml), or isotype control IgG. Values are means ± SD. **P<0.01 (n=6 per cell type).

Figure 4. Effect of parthenolide on MSC migration and adhesion. (A) Serum-starved and PTL-treated MSCs were added to the upper wells, and serum-free medium supplemented with PDGF-BB (10 ng/ml), HGF (30 ng/ml), MCP-1 (100 ng/ml), or SDF-1α (150 ng/ml) was added to the lower wells. Treatment with medium alone (DMEM/F-12) was a negative control and treatment with 30% FBS was the positive control. Values are expressed by relative number of cells compared to respective controls (without pretreatment with PTL). (B) To assess the inhibitory effect of PTL on NF-κB phosphorylation, PTL-treated MSCs were stimulated with recombinant TNF-α for 3 min, and cellular extracts were prepared for western blotting. (C) To monitor the inhibitory effect of PTL on NF-κB activation, immunofluorescent analysis of NF-κB p65 nuclear translocation was carried out as described in Materials and methods with an Alexa Fluor 488-conjugated specific antibody (Green). Actin filaments were labeled with Alexa Fluor 546-conjugated phalloidin (Red); nuclei were stained with DRAQ-5 dye (Blue). Objective magnification x
(D) Effect of PTL treatment on TNF-α-induced expression of adhesion molecules was analyzed by flow cytometry. PTL-treated MSCs were cultured with TNF-α (10 ng/ml) for 6 h. Cells were labeled with FITC-conjugated antibodies and analyzed by flow cytometry (filled histogram). Rat isotype antibodies IgG1 and IgG2a served as respective controls (open histograms). Values represent the percentage of positive cells after TNF-α stimulation, and values in parentheses represent the percentage of positive cells without TNF-α stimulation. (E) MSCs (1 x 10⁴) were added to ECs that had been cultured to confluence on fibronectin-coated 96 well plates. MSCs and ECs were pretreated with the following substances: parthenolide (5 µM), TNF-α (10 ng/ml), anti-VCAM-1, VLA-4 (10 µg/ml), or isotype control IgG. Values are expressed as means ± SD (n=6). *P<0.05 and **P<0.01.

Figure 5. In vivo imaging of NF-κB-suppressed MSC accumulation at tumor sites. (A) Luciferase-expressing MSCs were cultured with PTL for 6 h and luciferase assays were periodically performed. Values are expressed as means ± SD (n=4 each). (B) Cell viability of PTL-treated luciferase-expressing MSCs was also examined by XTT assays. Values are expressed as means ± SD (n=4 each). (C) Luciferase-expressing MSCs without PTL treatment were injected into tumor-bearing mice through the left ventricular cavity and IVIS imaging was periodically performed. Each data shown are from one representative experiment of eight performed. (D) Luciferase-expressing MSCs with PTL treatment were injected into tumor-bearing mice and IVIS imaging was periodically performed. Imaging was performed as described above. Each data shown
are from one representative experiment of eight performed. (E) Bioluminescent intensity at tumor sites was quantified using analysis software. The data are expressed as means ± SD (n=8 each). *P<0.05, **P<0.01 compared with a group of PTL (-) at the same time.
Figure 1

A. Cells (GFP) Tumor (RFP) Merge

MSCs

FBs

B. MSCs

Tumor

C. Tumor Fibroblasts

Days

D. x10^3 Photons/sec/cm^2

Days

MSCs

FBs

* * *
Figure 2

No. of migrated cells

Control
30% FBS
PDGF-BB
HGF
VEGF
MCP-1
SDF-1α
SW480-CM

MSCs

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

A

CD34 (Vasculature)

B

- control mice
- tumor-bearing mice

TNF-α [pg/mg tissue]

0 2 4 6

Liver Spleen Tumor

S

T

C

VLA-4

0.6

IC

VCAM-1

57.2 (4.5)

CD49d

24.5 (2.4)

CD29

82.8 (32.7)

D

MSCs

FBs

0.1

0.6

0.8

0.1

1.2

1.7 (0.8)

No. of adherent cells

0 2000 4000 6000 8000 10000

TNF-α IgG

- + + - + + -

Anti-VCAM1

- - + - - + -

Anti-VLA4

- - + - - + +
**Figure 4**

**A**

![Bar graph showing relative number of migrated cells for different treatments.](image)

**B**

![Western blots showing protein expression for different conditions.](image)

**C**

![Images of adherent cells stained with various markers.](image)

**D**

![Flow cytometry plots showing cell surface marker expression.](image)

**E**

![Graph showing number of adherent cells for different conditions.](image)
Figure 5

A: Corrected RLU over days after PTL treatment for PTL (-) and PTL (+)

B: [A_{490nm} - A_{650nm}] over days after PTL treatment for PTL (-) and PTL (+)

C: Images showing MSC and PTL treatment effects on tumor over days

D: Images showing MSC and PTL treatment effects on tumor over days

E: Photon counts per second per square centimeter over days for PTL (-) and PTL (+)
NF-κB activity regulates mesenchymal stem cell accumulation at tumor sites

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