Bone marrow-derived mesenchymal stem cells inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia after arterial injury in rats

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ABSTRACT

We investigated whether mesenchymal stem cell (MSC)-based treatment could inhibit neointimal hyperplasia in a rat model of carotid arterial injury and explored potential mechanisms underlying the positive effects of MSC therapy on vascular remodeling/repair. Sprague-Dawley rats underwent balloon injury to their right carotid arteries. After 2 days, we administered cultured MSCs from bone marrow of GFP-transgenic rats (0.8×10^6 cells, n = 10) or vehicle (controls, n = 10) to adventitial sites of the injured arteries. As an additional control, some rats received a higher dose of MSCs by systemic infusion (3×10^6 cells, tail vein; n = 4). Local vascular MSC administration significantly prevented neointimal hyperplasia (intima/media ratio) and reduced the percentage of Ki67 + proliferating cells in arterial walls by 14 days after treatment, despite little evidence of long-term MSC engraftment. Notably, systemic MSC infusion did not alter neointimal formation. By immunohistochemistry, compared with neointimal cells of controls, cells in MSC-treated arteries expressed reduced levels of embryonic myosin heavy chain and RM-4, an inflammatory cell marker. In the presence of platelet-derived growth factor (PDGF-BB), conditioned medium from MSCs increased p27 protein levels and significantly attenuated VSMC proliferation in culture. Furthermore, MSC-conditioned medium suppressed the expression of inflammatory cytokines and RM-4 in PDGF-BB-treated VSMCs. Thus, perivascular administration of MSCs may improve restenosis after vascular injury through paracrine effects that modulate VSMC inflammatory phenotype.

1. Introduction

Vascular injury caused by angioplasty provokes neointimal hyperplasia by inducing aberrant vascular smooth muscle cell (VSMC) proliferation and migration [1]. Although drug-eluting stents have significantly reduced restenosis rates, delayed arterial healing and late stent thrombosis have emerged as major concerns [2]. Thus, new tools and/or approaches are needed to better prevent restenosis after intervention. Adult bone marrow contains hematopoietic lineage stem/progenitor cells and endothelial progenitor cells as well as non-hematopoietic progenitor subsets commonly referred to as multipotent

stromal cells or mesenchymal stem/progenitor cells (MSCs). MSCs can be easily isolated from bone marrow and expanded in adherent culture systems. They are multipotent and capable of secreting pro-angiogenic and cytoprotective factors [3]. Accumulating evidence from animal studies shows that exogenous administration of cultured MSCs can ameliorate organ damage during disease and after injuries such as myocardial infarction or stroke [3,4]. Based on their attractive properties, MSCs are now recognized as tissue-repairing cells that may provide safe and effective cardiovascular cell therapies [5]. Previous reports from our group and others have demonstrated the therapeutic efficacy of MSC administration in animal models of cardiovascular

disease [6–10]. Recently, MSCs have drawn additional interest due to their ability to improve healing through modulation of vascular responses after injury. To date, however, mechanisms responsible for the observed vascular benefits of MSC treatment remain poorly understood.

Rapid re-endothelialization after vascular injury is important for restoring normal vascular function, reducing vascular inflammation, and preventing adverse remodeling and neointimal formation [11]. Thus, researchers employing cell-based approaches for vascular repair have largely focused on endothelial cells and their replacement [12–15]. By contrast, the present study investigates the role of VSCMs in vascular remodeling and the potential for MSC-based cell therapy to prevent neointimal hyperplasia through control of resident VSMCs.

2. Methods

2.1. Isolation and culture of MSCs from GFP-rat bone marrow

MSCs ubiquitously expressing enhanced green fluorescent protein (GFP) were isolated from the bone marrow of adult transgenic Sprague-Dawley rats and cultured as previously described [6]. In brief, mononuclear cells were purified from GFP-rat bone marrow by density centrifugation and resuspended in a complete culture medium (CCM) consisting of α-MEM (Life Technologies, Carlsbad, CA) with 20% fetal calf serum (FCS, Atlantic Biologicals, Norcross, Georgia), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine. The cells were plated into a 150 cm^2 plastic culture dish containing 20 ml of medium. They were then placed into a humidified incubator with 95% air and 5% CO_2 at 37 °C. After 24 h, non-adherent cells were removed by washes and the primary adherent cells were propagated in fresh culture medium.

2.2. Fluorescence activated cell sorting (FACS) analysis

Cultured MSCs were lifted with trypsin and re-suspended at 150,000 cells/ml in PBS. Pre-titered, phycoerythrin (PE) conjugated FACS antibodies and Isotype control antibodies (BD biosciences) were added to separate cell aliquots and incubated on ice for 30 min. After staining, the MSCs were centrifuged again and washed in PBS to remove unbound antibody. FACS was performed with a BD FACSAria cell sorter equipped with a 488 nm (blue) Coherent Sapphire laser. Gating by forward scatter and side scatter was used to exclude debris, cell doublets and aggregates. Flow cytometry plots were generated with FlowJo software (version 7.6.5.).

2.3. Rat carotid artery injury model and cell implantation

Eight-week-old male Sprague-Dawley rats were anesthetized with intraperitoneal injections of pentobarbital sodium. Right carotid arteries were denuded of endothelium with a 2F Fogarty balloon catheter that was introduced into the common carotid artery through the external carotid artery. The balloon was inflated three times, each time with immediate retraction of the catheter once full inflation was reached. After the procedure, the external carotid artery was ligated and the wound was closed.

The MSCs (passages 5–7) were plated and propagated in CCM with a change of medium every 4 days and harvested immediately before the implantation. Two days after the balloon injury, the rats were anesthetized again, the wounds were re-opened, and the injured arteries were exposed. Either cell suspension (0.8×10^6 cells, n = 10) or vehicle (control, n = 10) was administered (dropped) onto the adventitial sites. In another group, a higher dose of MSCs (3×10^6 cells, n = 4) was delivered systemically (tail vein) to rats with arterial injury. Tissue samples were obtained at day 14 after cell injection. For assessment of cell engraftment, another group of animals (n = 3) underwent the same experimental design and were sacrificed on day 3 after the therapy.

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Animal Care and Use Committee of Showa University approved the experimental protocol.

2.4. Histological study

Arteries were fixed with 4% paraformaldehyde and embedded in paraffin. Cross-sections of 4–5 μm in thickness were affixed to glass slides and examined by histological and immunohistochemical methods. Some of the cross-sections were stained with hematoxylin and eosin. Elastica van Gieson-stained sections were used for morphometry. In brief, images of the sections were recorded, digitized, and analyzed with Win ROOF image analysis software (MITANI Co., Fukui, Japan). For morphometric analysis, the following parameters were traced manually and measured by procedures previously described [16]: maximum intimal thickness, intimal area (area within the internal elastic lamina [IEL] minus the lumen area), and medial area (area of the external elastic lamina [EEL] minus the IEL area). The intimal/medial area ratio was also assessed.

2.5. Immunohistochemistry

Immunohistochemistry was performed as previously described [17]. Tissue sections were incubated with primary antibodies overnight at 4 °C, washed thrice in PBS, and incubated with peroxidase-labeled anti-rabbit, -mouse, or -goat antibody (Histofine Simplestain Max PO; Nichirei, Tokyo, Japan). The binding antibody was finally visualized by 3,3′-diaminobenzidine staining followed by counter-staining with hematoxylin. The following primary antibodies were used: SMemb (Yamasa, Tokyo, Japan), RM-4 (Trans Genic Inc., Hyogo, Japan), Ki67 (Abcam, MA, USA), and p27^{Kip1} (Dako, Glostrup, Denmark).

The sections were stained with anti- Ki67 and p27^{Kip1} antibodies to assess the proliferative activity of VSMCs in the vessel walls [16]. The total cell counts and total numbers of Ki67- or p27^{Kip1}-positive cells per high-power field were manually counted in three randomly selected regions per section (three sections per animal). Percentage of the Ki67- or p27^{Kip1}-positive cells in each section was calculated.

2.6. Immunofluorescence

Immunofluorescence was performed as previously described [10]. Tissue sections were incubated with anti-GFP (Life Technologies) and anti-α-smooth muscle cell actin (Dako, Glostrup, Denmark). The secondary antibodies used were raised against Alexa Fluor® 488 (1:200) and Alexa Fluor® 594 (1:400) (Invitrogen, CA, USA). Slides were mounted in Vectashield with DAPI (Vector Labs).

2.7. Preparation of conditioned medium

Conditioned medium (CdM) from the MSCs was prepared as previously described [6,8,9]. In brief, cells cultured to 70–80% confluence in 150 cm^2 dishes were washed with PBS (3 times), and incubated for 48 h with 15 ml of fresh α-MEM medium without serum or any supplements (N = 3). The culture supernatant was then collected, filtered, and stored at −80 °C.

2.8. Vascular smooth muscle cell proliferation assay

Rat aortic VSMCs (rVSMCs) were purchased from Cell Applications Inc. (San Diego, CA, USA) and cultured in smooth muscle cell basal medium (SmBM, Cell Applications) with 5% FCS and the appropriate supplementation. Cells at passages 3–5 were seeded onto 10 cm culture dishes (5×10^3 cells/cm^2) and incubated at 37 °C in 5% CO_2 for 72 h. After a wash with PBS, the rVSMCs were incubated for 24 h in serum- and supplement-free SmBM to arrest cell growth. Subsequently, the medium was changed to fresh α-MEM with 1%FCS and PDGF-BB

(20 ng/ml; R&D systems, MN, USA) or to the MSC-CdM with 1%FCS and PDGF-BB. The cells were harvested 24 h after the stimulation, and the cell counts were measured as described previously [18]. The cell number ratio was defined as cell number divided by the number at baseline. The cells harvested on day 3 after the stimulation were used for western blotting and real time RT-PCR analyses.

2.9. Western blotting

Western blotting was performed as previously described [19]. Cell lysates were subjected to SDS/PAGE (4–12% gradient gel). Proteins were transferred to ImmunoBlot™ PVDF membranes (0.2 μm; Life Technologies). After blocking, the membranes were incubated with
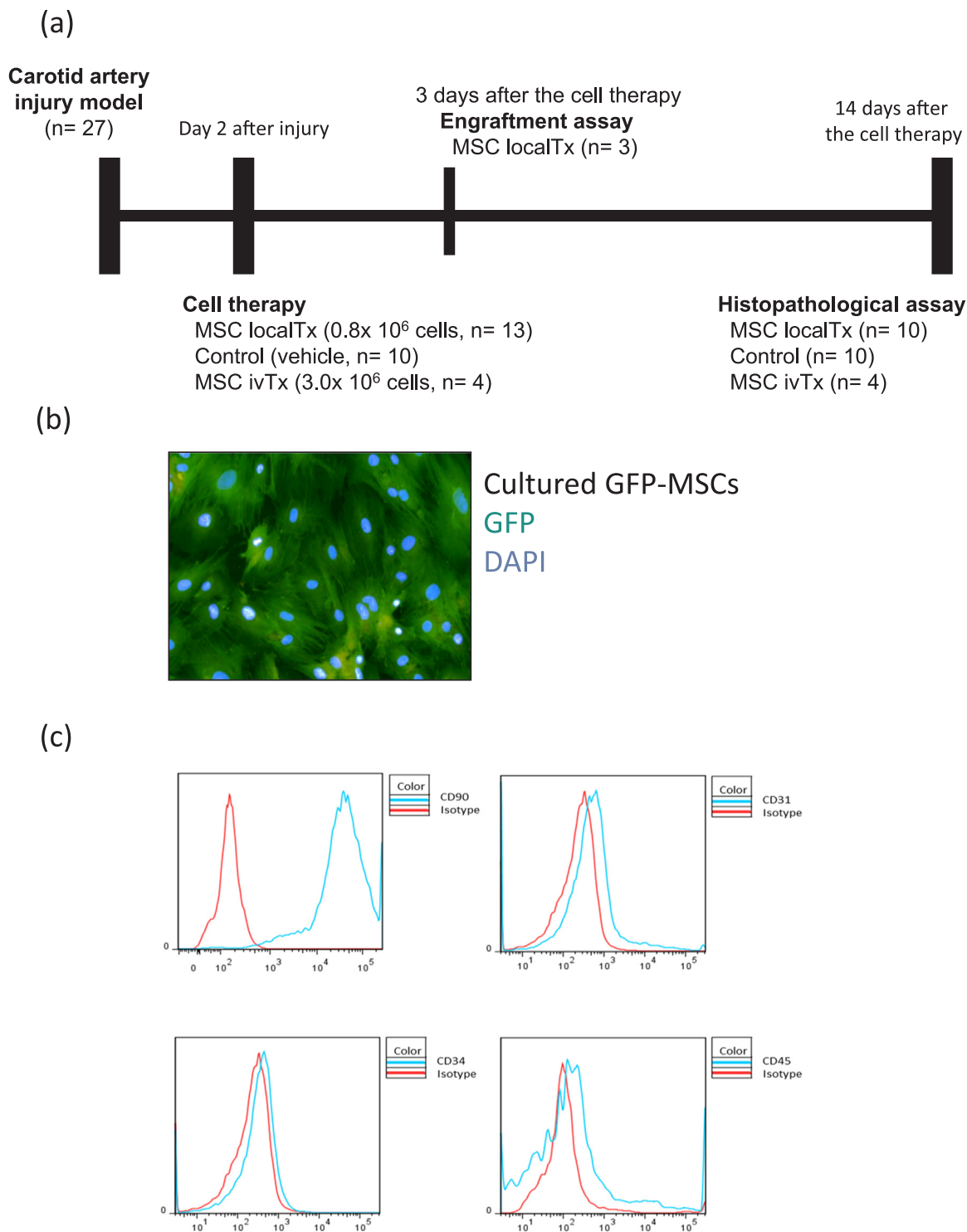

Fig. 1. *In vivo* experimental protocol and GFP-MSC characteristics. (a) Protocol of MSC implantation study. MSC localTx, local MSC administration onto the adventitial sites. MSC ivTx, systemic MSC administration via tail vein. (b) Cultured green fluorescence protein (GFP)-MSCs. Nuclei were stained with DAPI (blue). (c) Flow cytometric analysis for MSCs. GFP rat MSCs expressed the mesenchymal marker CD90 (Thy 1), but not markers of hematopoietic or endothelial cells (i.e. CD45, CD34, CD31). Blue = Cell surface epitope-specific antibodies, PE-conjugated and per-titered for FACS. Red = Non-specific isotype control antibodies, also PE-conjugated and per-titered for FACS.

primary antibodies against RM-4 (Trans Genic Inc.), p27^{Kip1} (Cell Signaling Technology, MA, USA), and β-actin (Abcam). Next, the membranes were incubated with secondary antibodies at 1:2000 dilutions (Santa Cruz Biotechnology), washed, and developed using ECL reagents (Santa Cruz Biotechnology).

2.10. Real time RT-PCR analysis

Total RNA was isolated from the rVSMCs using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) with a DNase (RNase-Free DNase Set; Qiagen) digestion step and quantified with a Nanodrop spectrophotometer. Quantitative real-time RT-PCR analysis was performed by methods similar to those previously described [6,17].

In brief, cDNA was synthesized and mixed with Taqman Universal Master Mix and each of the Taqman gene-specific probes/primers (Applied Biosystems). The PCR reaction was performed using the following Taqman PCR universal thermal cycling conditions defined by Applied Biosystems: 95 °C for 10 min, followed by 40 cycles of two-temperature PCR at 95 °C for 15 s for denaturing and 60 °C for 1 min for annealing and extension. Taqman probe/primers were used for interleukin (IL)-6 (il6, Rn01410330_m1), monocyte chemoattractant protein (MCP)-1 (Ccl2, Rn01456716_g1), intercellular adhesion molecule (ICAM)-1 (Icam1, Rn00564227_m1), cyclin D1 (Ccnd1, Rn00432360_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn99999916_s1). A comparative Ct method was used to assess the levels of each mRNA transcript relative to the level of the GAPDH mRNA transcript.

2.11. Statistical analysis

All data were expressed as mean ± SEM. Comparisons of parameters among three groups were performed by one-way ANOVA followed by post-hoc tests. The Student's t-test was used to compare differences between two groups. Probability values of < 0.05 were considered significant.

3. Results

3.1. Perivascular administration of MSCs from bone marrow inhibits neointimal formation without long-term MSC engraftment

Fig. 1a shows protocol of the MSC implantation study. GFP rat MSCs (Fig. 1b) expressed the mesenchymal marker CD90 (Thy 1), but not markers of hematopoietic or endothelial cells (Fig. 1c). The MSCs (0.8×10^6 cells) or vehicle was locally administered onto the adventitial site of the injured arteries 2 days after the injury was induced. In another group, a higher dose of MSCs (3×10^6 cells) was delivered systemically (tail vein) to rats with arterial injury.

Immunohistochemical assays to detect GFP were performed to reveal the extent of MSC engraftment in the rats with local MSC administration. We observed a few GFP-positive cells in the adventitia on day 3 after the administration (Fig. 2a) but detected no MSCs or differentiation into VSMCs, endothelial cells, or adventitial fibroblasts on day 14 after cell therapy (data not shown).

Morphometric analysis was performed to quantitatively evaluate the suppressive effects of the MSCs on neointimal formation after the arterial injury. By day 14 after treatment, local administration of MSCs significantly inhibited neointimal hyperplasia in carotid arteries (both the intima/media ratio and maximal intimal thickness) compared with controls (Fig. 2b, c). Notably, intravenous systemic administration of the MSCs did not reduce neointimal hyperplasia, even when the cells were infused at a 4-fold higher dose than that used for local administration.

3.2. Perivascular administration of MSCs alters VSMC phenotype and expression cell cycle regulators in VSMCs

To evaluate the proliferative activity of VSMCs in the injured arterial wall, we examined the levels of two proteins expressed during the cell cycle. Immunohistochemical assays performed with antibodies to Ki67 revealed the presence of Ki-67 protein during all active phases of the cell cycle (G$_1$, S, G$_2$, and mitosis), but not in the resting cells (G$_0$). Compared with the percentage of proliferating cells observed in vessels from the control group, perivascular administration of MSCs significantly reduced the percentage of Ki67 + proliferating cells in the neointima (Fig. 3a). In contrast, cells expressing p27^{Kip1}, a ubiquitous cyclin-dependent kinase inhibitor, significantly increased in the local MSC administration group than in the controls (Fig. 3b). Thus, the local MSC therapy inhibited cell cycle progression in the VSMCs of injured artery.

Neointimal VSMCs in an injured artery are dedifferentiated and phenotypically different from medial VSMCs in a normal artery. SMemb has been found in the VSMCs of fetal aortas and identified as a molecular marker for dedifferentiated VSMCs in vascular lesions [20]. RM-4 was originally identified as a molecular marker of rat inflammatory cells [21]. In the present study, SMemb and RM-4 were both detected in neointimal VSMCs (Fig. 3c), and both were expressed at reduced levels in the VSMCs from MSC-treated arteries relative to the levels expressed by VSMCs of controls. These results indicated that the MSC therapy may have suppressed or reversed the phenotypic changes typically observed in neointimal VSMCs after vascular injury.

3.3. Factors secreted by MSCs modulate the proliferation and phenotype of cultured VSMCs

Despite the lack of long-term MSC engraftment, local MSC therapy inhibited neointimal formation and altered the VSMC phenotype in injured arteries. In the absence of MSCs, paracrine action was likely to account for the benefits observed after MSC treatment. Therefore, we decided to conduct *ex vivo* experiments using MSC-CdM to test whether MSC-secreted factors could directly affect VSMC phenotype or proliferation.

Platelet-derived growth factor (PDGF)-BB is known both as a major mitogen for VSMC and also as a critical modulator of VSMC phenotype [22]. The rVSMCs exposed to PDGF-BB (20 ng/ml) propagated to significant levels relative to baseline, whereas the MSC-CdM significantly attenuated PDGF-BB-induced rVSMC proliferation (Fig. 4a). By western blotting of VSMC lysates, MSC-CdM treatment increased the expression of p27^{Kip1} protein. In addition to its inhibitory effect on VSMC proliferation, CdM treatment also suppressed the expression of RM-4 in PDGF-BB-treated rVSMCs (Fig. 4b).

Quantitative RT-RCR analysis confirmed that the MSC-CdM significantly repressed gene expression for IL-6, MCP-1, ICAM-1 and cyclin D1 in PDGF-BB-treated rVSMCs (Fig. 4c).

4. Discussion

Here we show that perivascular MSC administration can prevent neointimal progression after arterial injury, even in the absence of long-term MSC engraftment. In parallel, the local MSC therapy modified the VSMC phenotype. Our *ex vivo* experiments strongly support the hypothesis that the improvements in vascular remodeling/repair we observed after cell therapy were derived from the paracrine action of factors secreted by and/or released from transiently-engrafted MSCs.

Factors secreted by MSCs are known to alter the growth, differentiation, survival, and/or function of endogenous (resident) cells in various organs/tissues outside of the bone marrow, even though MSCs derive from a disparate tissue source [4,23]. We previously reported that the systemic administration of human MSCs into immunodeficient mice with myocardial infarction improved cardiac function via
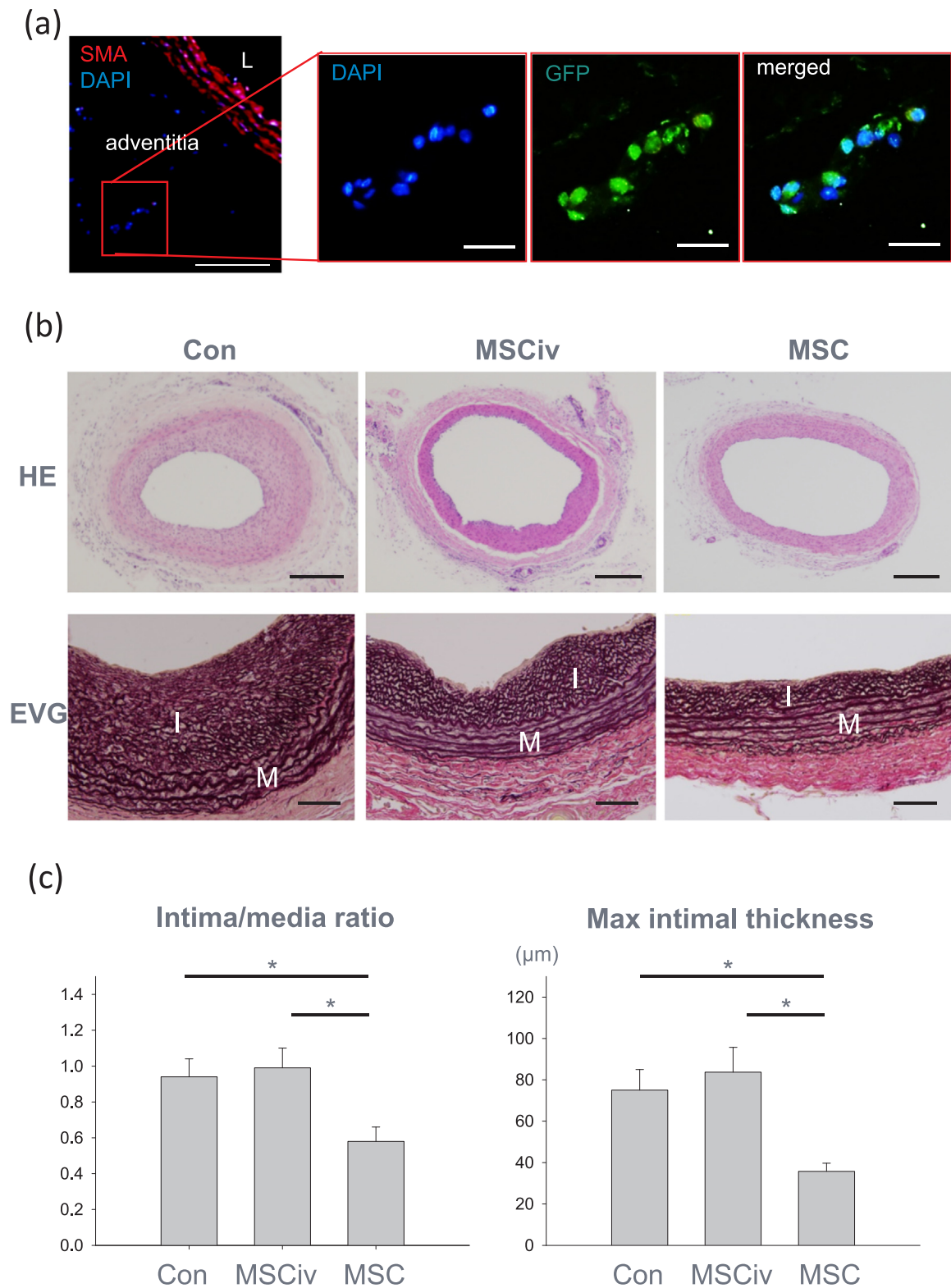
Fig. 2. Local MSC therapy in a rat vascular injury model. (a) Transient engraftment of MSCs without differentiation. A few GFP-positive MSCs (green) were detected in the adventitia 3 days after the perivascular administration of MSCs. Nuclei were stained with DAPI (blue). SMA (red), alpha-smooth muscle actin. DAPI, 4′,6-Diamidino-2-phenylindole. L, lumen of artery. Bar scale, left = 100 μm, right (3 panels) = 20 μm. (b) Prevention of neointimal formation by the perivascular MSC administration. Representative images of rat carotid arteries 16 days after the injury (14 days after the treatment). Con, controls. MSC, perivascular MSC administration. MSCiv, intravenous systemic MSC administration. I, intima. M, media. Bar scale, HE, hematoxylin-Eosin staining. EVG, elastica van Gieson staining. Bar scale, upper = 200 μm, lower = 50 μm. (c) Quantitative morphometric analyses. By day 14 after treatment, local perivascular administration of MSCs (MSC, n = 10) significantly suppressed neointimal hyperplasia (the intima/media ratio and the max intimal thickness) compared with controls (Con, n = 10). Intravenous MSC administration (MSCiv, n = 4) did not limit neointimal hyperplasia. *, p < 0.05.
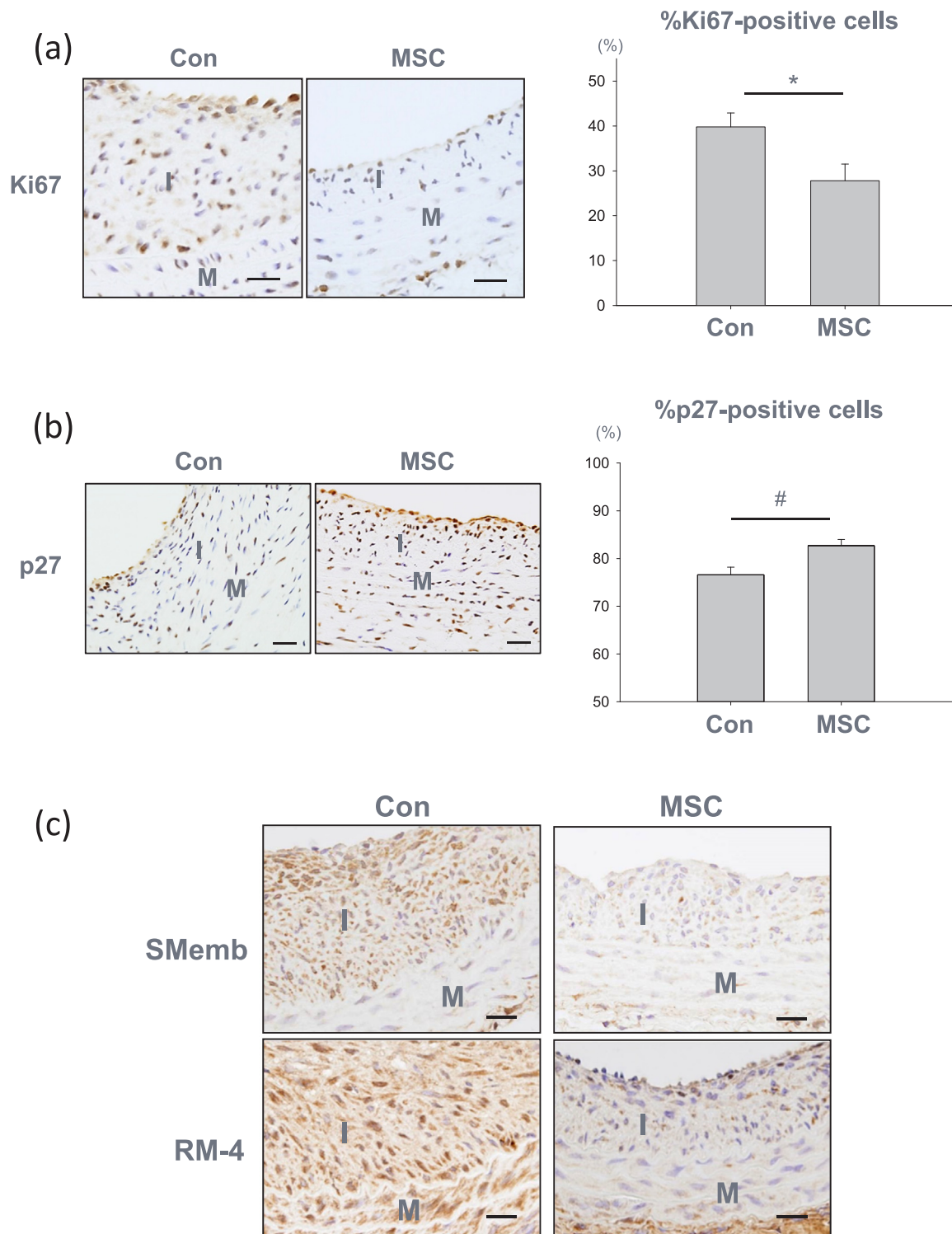
Fig. 3. Inhibition of VSMC proliferation by local MSC therapy. (a) Left images, Immunohistochemistry was performed with antibodies to Ki67 to evaluate cell proliferation. Brown staining in the nuclei indicate the presence of Ki67. Bar = 20 μm. Right graphs, Local MSC administration significantly decreased the number of proliferating VSMCs (Ki67-positive cells) in the arterial wall. Con, n = 10; MSC, n = 10. I, intima. M, media. *, p < 0.05. (b) Left images, Immunostaining for p27^{Kip1} protein. Brown staining in the nuclei indicate the presence of p27^{Kip1}. Bar = 20 μm. Right graphs, Local MSC administration significantly increased the number of p27^{Kip1}-positive VSMCs. Con, n = 10; MSC, n = 10. #, p < 0.01. (c) Phenotypic changes in VSMCs after local MSC therapy. Local MSC administration down-regulated the expression of SMemb and RM-4 in neointimal VSMCs. Brown staining indicates positive cells. SMemb, embryonic isoform of myosin heavy chain. RM-4, membrane protein of inflammatory cells. Bar = 20 μm.

paracrine effects [6]. Sequential studies from our group have also demonstrated that the MSC-secreted factors mediate angiogenesis, cardioprotection, and the activation of cardiac progenitors [7–10]. Notably, modification of the tissue microenvironment after injury through the paracrine activity of administered MSCs is currently recognized to play a more prominent role in MSC-mediated organ/tissue repair than does cell transdifferentiation [3].

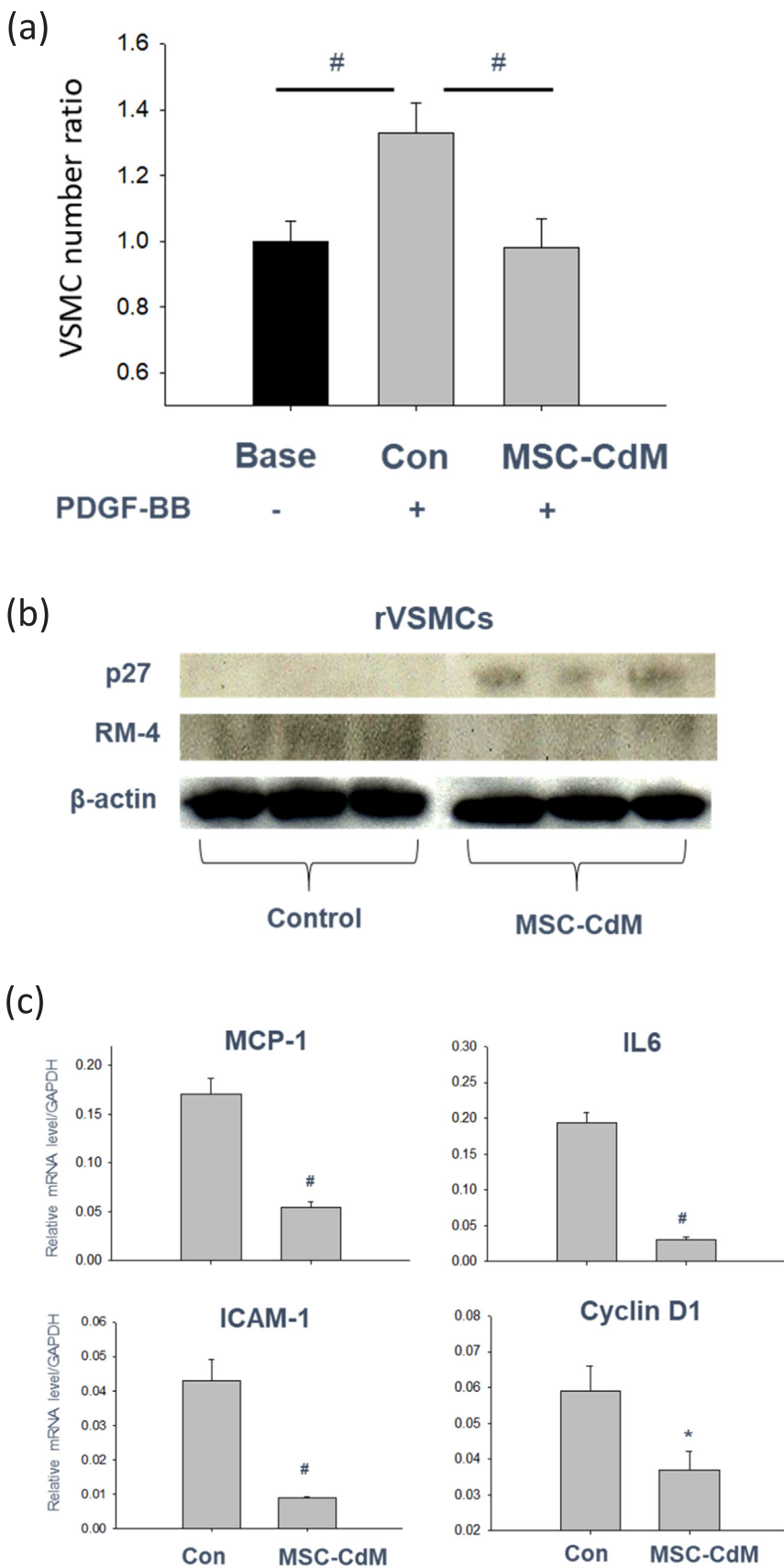In multiple animal models of injury, MSC-mediated

Fig. 4. Effects of factors secreted by MSCs on cultured-VSMC proliferation and phenotype. (a) Conditioned medium from MSCs (MSC-CdM) significantly attenuated the growth of rat VSMCs exposed to PDGF-BB. Base, baseline before the treatment. Con, the controls. n = 5 in each group. #, p < 0.01. (b) By western blot, we observed increased p27 expression and decreased RM-4 expression in PDGF-BB-stimulated rVSMCs treated with MSC-CdM compared with controls. Three independent experiments were performed. rVSMC, rat vascular smooth muscle cells. (c) Real-time RT-PCR analysis demonstrated that MSC-CdM significantly decreased gene transcription for IL-6, MCP-1, ICAM-1, and cyclin D1 in the rVSMCs exposed to PDGF-BB. n = 3 in each group. *, p < 0.05. #, p < 0.01.

immunosuppression has been shown to involve the release of IL-10 [24], nitric oxide [25], prostaglandin E2 [26], and tumor necrosis factor-α stimulated gene/protein 6 (TSG-6), by MSCs. TSG-6 has multifunctional anti-inflammatory properties [27] and was found to reduce the level of nuclear factor-κB signaling in macrophages, thereby modifying the cascade of proinflammatory cytokines normally released

after injury. Transplant vasculopathy is characterized by accelerated concentric intimal hyperplasia diffusely involving the entire coronary vascular bed and is a chronic inflammatory disease that develops in the setting of conventional immunosuppression regimens that are effective in the treatment of acute rejection to the myocardium. In a porcine transplant vasculopathy model, local delivery of autologous MSCs alleviates the transplant atherosclerosis by inducing allograft tolerance via immunomodulation in the vessel wall [28].

Mechanical vascular injury such as angioplasty also triggers local inflammation in the walls of blood vessels. In concert, macrophages and VSMCs release numerous cytokines and growth factors that include PDGF-BB; this results in increased smooth muscle cell proliferation, changes in VSMC phenotype, and neointimal hyperplasia [29]. Neointimal VSMCs are phenotypically different from medial VSMCs. Accumulating evidence shows that neointimal or dedifferentiated VSMCs exhibit an inflammatory cell phenotype [30]. In the present study we found that RM-4 was expressed by neointimal VSMCs of rat carotid arteries after injury and by rVSMCs simulated with PDGF-BB in culture. In rat macrophages and dendritic cells, RM-4 antisera was originally found to recognize a membrane protein component of endolysosomes [21]. In the presence of PDGF-BB, factors secreted by MSCs suppressed RM-4 expression in the rVSMCs, as well as transcription of the genes for key inflammatory cytokines such as IL-6, MCP-1, and adhesion molecules, such as ICAM-1. Our group recently reported reduced proliferation and migration in VSMCs exposed to TSG-6 [31]. Thus, the anti-inflammatory property of secreted factors from MSCs inhibited the rVSMC transformation to proliferative inflammatory phenotype, which might limit the neointimal hyperplasia.

Adventitia is a complex outermost layer of the vessel wall and a major site of immune surveillance and inflammatory cell trafficking, and actively participates in the initiation of vascular inflammation and neointimal formation following vascular injury [32]. Adventitia also have been shown to contain resident vascular progenitor cells express Sca-1 [33]. The adventitial Sca-1$^+$ cells contributed to atherosclerosis and neointimal formation after vascular injury via migration and differentiation into SMCs [33,34]. In the present study, perivascular MSC administration at the adventitial sites significantly prevented neointimal hyperplasia, whereas systemic MSC infusion did not. We thus speculate that part of the mechanisms underlying the benefits of the local treatment may be responsible for modulation of the immune response and progenitor cell activation in the adventitial tissue although further studies will be needed to address and confirm the phenomenon.

The present study is the first to demonstrate that MSC-secreted factors directly inhibit cell cycle progression and inflammatory phenotypic changes in VSMCs, acting much like sirolimus or anti-proliferative drugs of similar types that are coated onto stents. Importantly, our results suggest local administration of MSCs may provide an effective treatment that prevents or substantially reduces restenosis after angioplasty. In the future, we anticipate that stents seeded with MSCs, coated with concentrated MSC CdM, or coated with a defined combination of MSC-secreted factors could provide new, effective tools and unique "off-the-shelf" products that improve the lives of interventional cardiology patients.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.001.

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