Original Article

Human Mesenchymal Stromal Cells (MSCs) Reduce Neointimal Hyperplasia in a Mouse Model of Flow-Restriction by Transient Suppression of Anti-Inflammatory Cytokines

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Aim: Mesenchymal stromal cells from human bone marrow (hMSCs) were observed to produce therapeutic benefits in some models for cardiac and vascular injuries but their mode of action was not defined. We tested the effects of hMSCs in models for restricted vascular flow.

Methods: We made model for restricted vascular flow produced by permanent ligation of a carotid artery and injected hMSCs to clarify the effects of hMSCs to vascular lesions.

Results: Seven, 14, and 28 days after infusion of hMSCs into the cardiac left ventricle of the mice, there was a significant reduction in neointimal hyperplasia ($p < 0.05$). Seven days after administration of the hMSCs, macrophages infiltration into the ligated artery and serum levels of monocyte chemoattractive protein-1 (MCP-1/CCL-2) ($p < 0.05$) were reduced. However, no hMSCs were detected in the lesions by sensitive PCR assays. We then observed that the serum level of MCP-1 was a potential biomarker for the therapeutic effects of hMSCs in a mouse model for high-fat-diet.

Conclusions: These results indicated the administration of hMSCs decreased the initial and excess inflammatory responses to carotid artery ligation. The decrease in inflammatory response apparently decreased the subsequent neointimal hyperplasia.

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Key words; Stem/progenitor cells, Mesenchymal stem cells, MCP-1, Cellular engraftment, Biomarker

Introduction

Surgical manipulation or atherosclerotic damage to arteries frequently causes denudation of the endothelium lining the intima of arteries that activates an

inflammatory cascade, causing the proliferation of smooth muscles¹. The result is neointimal hyperplasia that reduces blood flow. Therefore, protection of the endothelium and the regulation of inflammatory responses are important for the treatment of arterial insults and manipulations^{2,3}.

Human adult stem/progenitor cells from bone marrow, referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (hMSCs), are of interest as potential therapeutic agents because they are easily isolated and expanded, and are capable of differentiating into multiple lineages⁴. The cells can migrate to engraft into injured tissues, and secrete a large number of cytokines⁵⁻⁷; therefore, use of MSCs as a potential therapeutic option to repair injured arteries is considered^{8,9}.

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Several reports indicate that administration of MSCs modulated re-endothelialization and inhibited arteriosclerotic changes by decreasing the proliferation of smooth muscle cells either by limiting tissue damage or by MSCs differentiating to endothelial cells^{8, 10, 11}. In contrast, one report indicated that MSCs aggravated postangioplasty aortic restenosis in rats¹²; however, none of the reports established the mode of action of the MSCs.

In the present study, we first demonstrated that hMSC administration inhibited neointimal hyperplasia in a carotid artery ligation model, apparently by decreasing the initial inflammatory response to the lesion. Next, we observed the serum level of monocyte chemoattractive protein-1 (MCP-1/CCL-2) as a potential biomarker for the therapeutic effects of hMSCs in a mouse model of a high-fat diet.

Method

Preparation of Cells

hMSCs from normal healthy donors were obtained from the Tulane Center for the Preparation and Distribution of Adult Stem Cells (http://www.som.tulane.edu/gene_therapy/distribute.shtml). The hMSCs are now available from mscs@medicine.tamhsc.edu and were prepared as previously described¹³ with protocols approved by the Institutional Review Board. In brief, bone marrow aspirates were taken from the iliac crest of healthy adult donors. Nucleated cells were isolated using a density gradient (Ficol-Paque; Amersham Pharmacia Biotech, Milwaukee, WI, USA) and resuspended in complete hMSC medium: α -MEM (GIBCO/BRL, Grand Island, NY, USA); 20% FBS (lot selected for rapid growth; Atlanta Biologicals, Norcross, GA, USA); 100 units/mL penicillin (GIBCO/BRL); 100 μ g/mL streptomycin (GIBCO/BRL); and 2 mM L-glutamine (GIBCO/BRL). Cells (4 to 13×10^7) were then plated in 20 mL medium in a 180-cm² culture dish and incubated at 37°C with 5% humidified CO₂. After 24 hours, nonadherent cells were removed. Adherent cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and incubated with fresh medium. After 5 to 7 days, the cells were harvested with 0.25% trypsin/1 mM EDTA for about 5 min at 37°C and then replated at approximately 3 to 100 cells/cm² in an inter-connecting flask system (Cell Factory; Nunc, Rochester, NY, USA). When the cultures reached 70% confluence, the cells (passage-1) were harvested with trypsin/EDTA, resuspended at 1×10^6 cells/mL in α -MEM with 5% dimethyl sulfoxide and 30% FBS, frozen in 1-mL aliquots overnight at -80°C, and then stored in liquid

nitrogen. For cell expansion, a frozen vial of passage-1 hMSCs was thawed, plated in 25 mL complete hMSC medium in a 180-cm² culture dish (Nunc), and incubated at 37°C with 5% humidified CO₂. After 24 hours, the medium was removed and adherent, viable cells were washed twice with PBS, harvested with 0.25% trypsin/1 mM EDTA, and replated at 100 cells/cm² in hMSCs medium and incubated with a medium change every 3-4 days. The cells (passage-2) were incubated until they reached 70% confluence (approximately 7 days), at which time they were harvested with trypsin/EDTA. The hMSCs were expanded again as above and used here as P3 cells. For transplantation, the harvested P3 cells were washed with PBS, centrifuged and resuspended in Hank's balanced salt solution (HBSS) or PBS at $1 \times 10^6/150 \mu$ L (about 6,700 cells/ μ L). The epitope profile of cells from both passages was essentially the same: negative for hematopoietic markers (CD34, CD36, CD117, and CD45), and positive for CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD59 (>99%), CD90 (>99%), CD105 (>99%), and CD166 (>99%).

Flow-Restriction Vascular Injury

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and all animal protocols were approved by the Animal Care and Use Committee of Institutional Tulane University Health Sciences Center. Eight- to ten-week-old male Balb/c Snu/nu mice, weighing between 20 and 25 g, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The experimental protocol was as previously reported¹⁴. The mice were anesthetized with a solution of ketamine (80 mg/kg; Fort Dodge Laboratories, Inc. Sligo, Ireland) and xylazine (5 mg/kg; Lloyd Laboratories Walnut, CA, USA) injected intraperitoneally. A flow-restriction vascular injury was caused by ligating the bifurcation of the left common carotid artery. The distal left common carotid artery and its bifurcation into the external and internal carotid arteries were exposed using minimal dissection. The common carotid artery was completely ligated just proximal to the bifurcation with a 6-0 silk suture. All animals recovered and showed no symptoms of a stroke. Immediately, 6 days, and 13 days after ligation, the mice were anesthetized with a solution of ketamine (80 mg/kg) and xylazine (5 mg/kg) injected intraperitoneally, and injected into the cardiac left ventricle with either 150 μ L HBSS (HBSS group) or 10^6 hMSCs in 150 μ L HBSS (hMSC group). Mice were sacrificed using intraperitoneal administration of an overdose of

Fig. 1. Time course of carotid artery ligation model.

Immediately, 6 days, and 13 days after carotid ligation, the mice were injected into the cardiac left ventricle with either 150 μ L HBSS or 10^6 hMSCs in 150 μ L HBSS. Mice were sacrificed at 7, 14 and 28 days.

Neointimal hyperplasia continued to increase for up to 28 days after ligation.

Transverse sections were stained with hematoxylin-eosin.

Bar: 20 μ m. Arrowheads indicate internal elastic lamina (IEL).

pentobarbital (250 mg/kg) at 7, 14 and 28 days ($n=5$ to 6 in each group) (**Fig. 1**). At the time of sacrifice, blood was collected from the right cardiac ventricle to obtain serum for ELISA. The mice were then perfused with 0.9% NaCl solution, followed by perfusion fixation with 4% paraformaldehyde in PBS (pH 7.4). The carotid arteries were carefully excised, fixed in 4% paraformaldehyde overnight 4°C, and embedded in paraffin. Cross sections (5 μ m) were stained with hematoxylin and eosin or Masson trichrome for morphometric analysis.

High-fat Diet Model

Six- to eight-week-old male Balb/c Scl-nu/nu mice, weight between 20 and 25 g, were purchased from Jackson Laboratory. Mice were placed on a high-fat “western-type” diet for 12 weeks¹⁵. The western diet consisted of 42% of total calories from fat and 0.15% of cholesterol. At 12 weeks, the mice were

anesthetized by an intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine solution and were injected into the left ventricle of the heart with either 150 μ L PBS ($n=16$) or 10^6 hMSCs in 150 μ L PBS ($n=16$). For the detection of the inflammatory cytokines in mouse serum, blood was collected from the vascular bundle located at the rear of the jaw bone using a needle (Goldenrod Animal Lancets; MEDIPoint, Inc., Mineola, NY, USA) before and 24 hours after administration of either PBS or hMSCs (**Fig. 2**). Serum from four mice in each group of 16 mice was pooled into one sample and assayed using ELISA.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized, incubated with 0.3% hydrogen peroxide in methanol for 10 min, and blocked with 5% horse serum for 5 min. Next, the sections were incubated with an anti-mouse macrophage antibody (F4/80;

Fig. 2. Time course of high-fat diet model.

Mice were placed on a high-fat "western-type" diet for 12 weeks and injected into the left ventricle of the heart with either 150 μ L PBS or 10^6 hMSCs in 150 μ L PBS. Blood was collected before and 24 hours after administration of either PBS or hMSCs.

AbD Serotec, Raleigh, NC, USA) or α -smooth muscle actin antibody (SMA) (1A4; DAKO, Carpinteria, CA, USA) for 1 hour at room temperature in a moisture chamber. The slides were incubated with Alexa 488-labeled goat anti-mouse IgG (green; Molecular Probes, Eugene, OR, USA; 1:400 dilution) as the secondary antibody for 90 min with shading from light. Negative controls used non-immune serum instead of the primary antibody. The nuclei were stained with DAPI (1:10000). Sections were observed with a fluorescence light microscope (Olympus, Tokyo, Japan).

For detection of human cells, the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA, USA) was used. Paraffin-embedded tissue sections were deparaffinized, incubated with 0.3% hydrogen peroxide in methanol for 10 min, blocked with 5% horse serum, and incubated with anti-human nuclei antibody (235-1; Millipore, Billerica, MA, USA) for 1 hour at room temperature in a moisture chamber. The slides were washed with PBS, and incubated with biotinylated secondary antibodies (1:2,000; Vector Laboratories) for 10 min followed by avidin-biotin horseradish peroxidase. The sections were visualized with diaminobenzidine substrate.

Histological Analysis

Morphometric analysis of the sections was performed as previously described¹⁶⁻¹⁸. Blocks of the arteries were cut into 5 μ m transverse sections and stained with hematoxylin-eosin. Four different sections from 5 to 6 animals in each group were digitized to trace the entire lumen, the internal elastic lamina, and external elastic lamina using Image J image analysis software (NIH image). The intima/media ratio was averaged for each artery.

The ratio of anti-macrophage antibody-positive cells to all DAPI-positive nucleus cells in the neointima after vascular ligation was measured by count-

ing the number of neointimal cells with DAPI-stained nuclei. The cells were counted on four F4/80-stained sections visualized under high-power magnification from each of the 5 to 6 animals in each group.

hAlu Real-Time PCR Analysis

Seven, 14, and 28 days after carotid artery ligation, the mice were sacrificed and bilateral carotid arteries, liver, spleen, and lung were carefully isolated. The tissues were snap-frozen in liquid nitrogen and stored at -80°C . Genomic DNA from artery samples was extracted ($n=5$ to 6 in each group; DNeasy; Qiagen, Valencia, CA, USA), and total DNA was assayed by UV absorbance. Real-time PCR was performed with 50 ng target DNA, human Alu-specific primers, and a fluorescent probe¹⁹ on an automated instrument (Model 7700; Applied Biosystems, Foster City, CA, USA). The primers were: Alu forward, 5'-CAT GGT GAA ACC CCG TCT CTA-3'; Alu reverse, 5'-GCC TCA GCC TCC CGA GTA G-3'; Probe, 5'-FAM-ATT AGC CGG GCG TGG TGG CG-TAMRA-3' (Integrated DNA Technologies, Coralville, IA, USA). Standard curves were prepared by adding 1×10^2 to 2.5×10^4 hMSCs to samples of the carotid arteries, liver, spleen, lung from uninjured mice. All assays were performed in triplicate.

ELISAs

Serum was assayed using ELISA specific for mouse MCP-1, MCP-3, CRP and VCAM-1 (R & D Systems, Minneapolis, MN, USA). Quantification was performed by measuring absorbance at 450 nm and then subtracting out baseline absorbance at 540 nm using an automated plate reader (Fluostar Optima; BMG Labtech Inc. Cary, NC, USA). Standard curves were generated using recombinant mouse MCP-1, MCP-3, CRP and VCAM-1 (R & D Systems).

Fig. 3. Inhibition of neointimal hyperplasia by hMSCs administration after vascular ligation.

(A) hMSC administration inhibited neointimal hyperplasia after vascular ligation. (B) The intima/media ratio of the carotid arteries was calculated after vascular ligation. The neointimal area was significantly smaller in the hMSC-treated group than in the control group throughout the experiment ($p < 0.05$).

Upper panels: low magnification, lower panels: high magnification.

Transverse sections were stained with hematoxylin-eosin.

Bar: 20 μ m. Arrowheads indicate IEL.

Statistical Analysis

All data are expressed as the mean $\pm$ SE. Statistical comparisons were made using Student's *t*-test for unpaired samples. $P < 0.05$ was considered significant.

Results

Administration of MSCs Decreased Neointimal Hyperplasia After Carotid Artery Ligation

First, we confirmed the time course of neointi-

Fig. 4. Inhibition of macrophages in neointima by hMSC administration after carotid artery ligation.

(A) hMSC administration decreased the number of F4/80 positive-inflammatory cells accumulating in neointima 7 days after vascular ligation. Red cells were positive for F4/80 antibody. Green cells were positive for α -SMA and blue is DAPI. (B) We calculated the number of F4/80-positive cells accumulating in the neointima of ligated arteries 7 days after vascular ligation. Significantly fewer inflammatory cells were found in hMSC-treated samples than in the control ($p < 0.05$).

Upper panels: low magnification, lower panels: high magnification.

Bar: 20 μ m. Arrow indicates F4/80-positive cells. Arrowheads indicate IEL.

mal hyperplasia after carotid artery ligation in the mouse model. Neointimal hyperplasia continued to increase for up to 28 days after ligation (**Fig. 1**) as previously described¹⁵. Next, to detect the efficacy of

hMSC injection, the intima/media ratio was assayed in the model. At 7 days after ligation, neointimal hyperplasia was much less in the hMSC group (intima/media ratio 0.081 ± 0.004 , $n=6$) than in the

Fig. 5. Serum MCP-1 after ligation of the left common carotid artery.

hMSC administration clearly inhibited the expression of MCP-1 in mouse serum in the early phase. The concentration of MCP-1 protein was significantly lower in the hMSC-treated group than in the control group 7 days after ligation ($p < 0.05$). There were no significant differences in each group 14 and 28 days after ligation. # $p < 0.05$ vs. HBSS injection as a control.

HBSS group (intima/media ratio 0.123 ± 0.006 , $n = 5$, $p < 0.05$) (**Fig. 3A, B**). Neointimal hyperplasia was increased in the control at 14 and 28 days but continued to be lower in the hMSC-treated group. The values at 14 days were 0.104 ± 0.003 ($n = 6$) in the hMSC-treated group vs. 0.379 ± 0.045 in the control ($n = 6$, $p < 0.05$). At 28 days, the values were 0.149 ± 0.006 vs. 0.423 ± 0.048 ($n = 5$, respectively, $p < 0.05$).

hMSCs Decrease Neointimal Hyperplasia without Significant Long-Term Engraftment

To determine whether hMSCs engrafted into the vascular lesion, DNA extracted from the ligated carotid arteries was assayed with a sensitive real-time PCR assay for human *Alu* sequences (h*Alu*). Standard curves were prepared by adding 1×10^2 to 2.5×10^4 hMSCs to samples of carotid arteries from uninjured mice as previously described¹⁶⁾ (standard curve liner regression; $y = -0.2902x + 8.6088$, $R^2 = 0.9981$; supplemental figure). No significant *Alu* sequences were detected in 50 ng DNA assayed 7, 14, and 28 days after ligation in mice administered hMSCs. Assuming an average DNA content of 5 pg per cell, the results indicated that there was less than one human cell per 10,000 cells in the samples. hMSCs were not detected in other organs but in the lung 7 days after ligation (249.7 ± 79.9 cells). In addition, sections of the arteries were assayed by immunohistochemistry using human specific anti-nuclei antibody. Again, no human cells were detected in carotid arteries (supplemental figure).

Administration of hMSCs Decreased Macrophage Infiltration 7 Days After Carotid Artery Ligation

To evaluate the local inflammatory response, we performed immunohistochemistry in each section from mouse carotid arteries using an antibody to the macrophage marker, F4/80. At 7 days after the injury, F4/80-positive inflammatory cell accumulation on the neointimal hyperplasia was higher in the HBSS group ($11.4 \pm 0.22\%$ of cells, $n = 5$) than in the hMSC-treated group ($8.01 \pm 0.36\%$, $n = 6$, $p < 0.05$) (**Fig. 4A, B**). At 14 and 28 days, macrophages were rare in both the HBSS and hMSC-treated groups (not shown).

Serum MCP-1 as a Biomarker for the Effects of hMSCs in a Model of Flow Restriction

We next tested serum MCP-1 as a biomarker for the effects of hMSCs in a mouse model of restricted arterial flow. Seven days after ligation of the left common carotid artery, the serum level of MCP-1 protein was elevated in HBSS mice with ligation and was significantly lower in mice in which hMSCs were infused immediately after surgery and on day 6 (100.7 ± 5.15 pg/mL, $n = 5$, vs. 56.1 ± 1.86 pg/mL, $n = 6$, $p < 0.05$) (**Fig. 5**). After 14 and 21 days, the levels decreased in the HBSS group with ligation and there was no difference from the control group that was infused with hMSCs on days 0, 6 and 13 (43.2 ± 0.95 vs. 47.2 ± 1.83 , $n = 6$ and 28.2 ± 1.02 vs. 26.3 ± 0.50 , $n = 5$, respectively, n.s.).

Fig. 6. Inhibition of MCP-1 protein in serum by hMSC administration to mice placed on a high-fat diet.

(A) Serum MCP-1 in mice before (baseline) and 24 hours after administration of hMSCs to mice that had been on the diet for 12 weeks. (B) Serum MCP-3 in the same mice. (C) Serum CRP in the same mice. (D) serum VCAM-1 in the same mice. ## $p < 0.01$. # $p < 0.05$.

Biomarker for the Effects of hMSC Administration in the High-fat Model

In the second experiment, attempts were made to establish a biomarker for the effects of hMSCs in a mouse model of a high-fat diet. For this purpose, 10^6 hMSCs were infused into the cardiac left ventricle of mice that had been fed a high-fat diet for 12 weeks, and the sera were assayed with ELISA to evaluate changes in the inflammatory response (**Fig. 2**). The level of MCP-1 in mouse serum was not different in each group at baseline and 24 hours before injection (concentration: 87.8 ± 3.04 pg/mL in PBS group, 79.8 ± 6.96 pg/mL in hMSCs group, 4 samples, respectively, n.s.). It was higher in mice on the diet for 12 weeks who were injected with PBS 24 hours beforehand (237.3 ± 23.7 pg/mL, $n=4$) but was lower in mice injected with hMSCs (80.3 ± 6.96 pg/mL, $n=4$, $p < 0.01$) (**Fig. 6A**). Moreover, hMSC administration also decreased the serum level of MCP-3 protein. The concentration of MCP-3 protein in mouse serum was not different in each group at baseline (227.5 ± 13.9 pg/mL in PBS group, 212.5 ± 3.76 pg/mL in hMSC group, 4 samples, respectively, n.s.), but was significantly lower in mice treated with hMSCs ($282.3 \pm$

37.6 pg/mL vs. 602.5 ± 74.0 pg/mL, $n=4$, $p < 0.05$) (**Fig. 6B**); however, administration of hMSCs did not decrease the serum level of CRP (before 6.7 ± 0.18 μ g/mL in PBS group and 6.1 ± 0.03 μ g/mL in hMSC group; after 6.5 ± 0.16 μ g/mL in PBS group and 6.9 ± 0.08 μ g/mL in hMSC group, $n=4$, n.s.). Also, administration of hMSC did not decrease the serum level of VCAM-1 (before 1612 ± 20.3 ng/mL in PBS group and 1558 ± 44.6 ng/mL in hMSC group; after 1573 ± 83.7 ng/mL in PBS group and 1495 ± 26.6 ng/mL in hMSC group, $n=4$, n.s.) (**Fig. 6C, D**).

Discussion

We indicated that hMSCs reduced neointimal hyperplasia after carotid ligation through the decrease of MCP-1 in mouse serum and inflammation of carotid arteries. Next, we confirmed that MCP-1 might be a potential biomarker which hMSCs could modulate in a model of a high-fat diet.

The present study demonstrated that systemic administration of hMSCs decreased neointimal hyperplasia in a mouse model without significant long-term engraftment of the cells into the lesion. Instead, the

Fig. 7. Schematic illustration of the effects of hMSCs on ligated carotid artery (see Discussion).

therapeutic benefit of the cells was probably explained by the modulation of inflammatory responses (**Fig. 7**), as demonstrated by decreased macrophage infiltration and serum MCP-1 7 days after ligation of the artery and administration of the cells. Similar reports of therapeutic improvement without significant engraftment of MSCs have now been reported in a number of animal models²⁰⁻²². The results suggest that the cells have a “touch and go” effect²³ by suppressing inflammatory and immune reactions^{24, 25}.

The site of the anti-inflammatory actions of hMSCs in many situations is not apparent. In these experiments, the hMSCs were infused into the cardiac ventricle to minimize trapping of the cells in the lung, which occurs after intravenous infusion²⁶⁻²⁸. This strategy was used previously when administering hMSCs to immunodeficient mice made diabetic by the administration of streptozotocin²⁹; therefore, it is possible that hMSCs transiently engrafted in the arterial lesion to exert their anti-inflammatory effects. However, the decrease of serum MCP-1 raises the possibility that the anti-inflammatory effect was produced by hMSCs that were trapped in the lung or peripheral capillaries and that secreted one or more anti-inflammatory factors.

MCP-1 is an important chemokine that regulates the migration and infiltration of inflammatory cells, such as monocytes/macrophages. The expression of MCP-1 is pro-inflammatory, directly related to the extent of atherosclerotic lesions in arteries. For example, anti-MCP-1 gene therapy limited the progression of established atherosclerosis in an animal vascular injury model³⁰. MCP-3 is a CC chemokine that functions by activating monocytes via the specific chemokine receptor CCR2. It is known that CCR2 is shared

with MCP-1, and MCP-3 also plays a role in the pathogenesis of restenosis or atherosclerosis³¹. We observed that the serum level of MCP-1 was decreased in mouse serum 7 days after administration of hMSCs. Our results also indicated that serum levels of MCP-1 and MCP-3 were significantly decreased 24 hours after hMSC administration in a high-fat diet model. Because MCP-1 has direct proatherogenic properties, in addition to its effects on the amplification of the inflammatory cascade, it could have played a major role in inhibiting neointimal hyperplasia after surgery in this experimental model.

MSCs are widely known to have pleiotropic effects, such as cytoprotection and anti-apoptosis. MSCs produce an anti-immune or immune-suppressive response, consistent with numerous reports that the cells have similar effects in culture, in animal disease models, and in patients with graft-vs.-host disease^{6, 7, 32-34}. These anti-inflammatory effects are consistent with those in a mouse model of lung injury^{35, 36}. Moreover, MSCs also secrete various growth factors, such as nitric oxide, vascular endothelial growth factor, and placental growth factor-1^{5, 37}, which regulate vascular remodeling. Taken together, our results suggest that hMSCs could be useful and safe tools for restenosis or atherosclerosis therapy.

At the same time, it is important to note that inconsistent results have been reported with MSCs. For example, one report indicated that hMSCs might increase neointimal hyperplasia through the excessive repair process of the vessel *in vivo*³⁸. Also, another group reported the unexpected observation that MSCs aggravated plaque stability through the accumulation of inflammatory cells in a rat angioplasty model¹². This indicates that hMSCs may accumulate and trans-

differentiate not only into endothelial progenitor cells, but also putative smooth muscle progenitor cells, resulting in an increased intima-media ratio. The discrepancies with our own results are probably explained by variables in the experimental conditions, such as subtle differences in the animal models, as well as differences in the protocols for preparing and expanding the MSCs in culture. The hMSCs employed in the experiments reported here were prepared with a standardized low-density protocol that provides cultures more enriched for early progenitor cells than the commonly employed confluent cultures. The cells from the low-density cultures express higher levels of epitopes linked to cell motility, are more clonogenic, differentiate more efficiently *in vitro*, and are less prone to produce lethal emboli after intravenous infusion into mice²⁷⁾. Some of the discrepancies in published reports might be resolved by using serum markers for inflammation, such as MCP-1. Serum levels of MCP-1 and other markers should also be useful in monitoring the responses of patients in clinical trials with MSCs.

Our immunohistological data demonstrated that neointimal hyperplasia in the early phase is composed of inflammatory cells without human-derived cells. This suggests that inflammatory cells affect the process of neointimal formation and elevate the intima/media ratio after carotid artery ligation. Therefore, the inhibition of inflammatory reactions via the endogenous or paracrine effects of hMSC administration may lead to stability of the vascular region and decrease the neointimal portion (**Fig. 7**). A further hypothesis is that hMSCs may mobilize more endogenous stem cells from bone marrow to the peripheral circulation, and the mobilized cells accumulate on the injured side of the vascular luminal where they may modulate both systemic and local inflammatory responses.

In conclusion, the results demonstrated that hMSC administration after ligation of the common carotid artery in mice reduced the initial increase of the serum level of MCP-1 and neointimal proliferation, apparently by reducing the initial excessive inflammatory response. In addition, administration of hMSCs to mice that are a model of a high-fat diet reduced serum levels of two pro-inflammatory proteins, MCP-1 and -3; therefore, the results suggested that serum levels of MCP-1 or -3 might be useful biomarkers for the anti-inflammatory effects of MSCs. The overall effect of hMSCs was to heal the vascular lesion by secreting endogenous and paracrine factors that modulated the inflammatory response without evidence of significant long-term engraftment of the cells.

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