Original Research

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Autologous Mesenchymal Stem Cells Foster Revascularization of Ischemic Limbs in Systemic Sclerosis

A Case Report

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Background: Mesenchymal stem cells can differentiate into endothelial cells and participate in angiogenesis in adults. In experimental models of acute myocardial infarction, mesenchymal stem cells led to the recovery of cardiac function through the formation of a new vascular network.

Objective: To describe treatment with intravenous infusions of expanded autologous mesenchymal stem cells in 1 patient with critical limb ischemia due to systemic sclerosis.

Design: Case report.

Setting: The rheumatology unit at the University of Florence, Florence, Italy.

Patient: A woman, aged 34 years, with systemic sclerosis who developed acute gangrene of the upper and lower limbs.

Intervention: 3 intravenous pulses of expanded autologous mesenchymal stem cells.

Measurements: Angiography, skin histopathology, and immunohistochemistry. **Results:** Areas of necrotic skin were reduced after the first mesenchymal stem-cell infusion. After the third infusion, angiography showed revascularization of the patient's extremities. Skin section analysis revealed cell clusters with tubelike structures, and angiogenic factors were strongly expressed.

Limitation: Causality cannot be established by a single case.

Conclusion: In patients with systemic sclerosis who have severe peripheral ischemia, intravenous infusion of expanded autologous mesenchymal stem cells may foster the recovery of the vascular network, restore blood flow, and reduce skin necrosis.

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Systemic sclerosis is a vascular disease, but little is known about the mechanisms that initiate endothelial and microvascular injury; prevent its repair; and lead to ischemia, loss of angiogenesis, digital ulcers, or occasionally gangrene (1).

Recent research has demonstrated that mesenchymal stem cells can differentiate into endothelial cells in vitro (2) and participate in the formation of new blood vessels in adults (3). In experimental models of acute myocardial infarction, intramyocardial injection of mesenchymal stem cells restored cardiac function through the formation of a new vascular network and arteriogenesis (3). Autologous implantation of bone marrow–derived mesenchymal stem cells into chronic nonhealing ulcers has been shown to accelerate the healing process and significantly improve clinical parameters (4).

Although this treatment has shown promise in some clinical trials and preclinical animal models, conflicting results have been reported (5). This evidence prompted us to use autologous mesenchymal stem-cell infusion to treat a

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case of extensive critical limb ischemia that complicated systemic sclerosis, in an attempt to recover the vascular network of the limbs.

CASE REPORT

A woman, aged 34 years, was admitted to the rheumatology unit of the University of Florence, Florence, Italy, in 2007 for the onset of large necrotic areas that rapidly extended from her digits to her forearms and legs (Figure 1, A). The patient had had systemic sclerosis since 2004 but had no cardiovascular risk factors. In the past 3 years, she had received corticosteroids and azathioprine and reported having Raynaud phenomenon but had never had digital ulcers. After drug therapy was stopped, she developed fever, arthromyalgia, acute-phase reactant elevation, and rapidly progressive finger ischemia. At admission, her erythrocyte sedimentation rate and C-reactive protein level were elevated. She was positive for antinuclear and anticentromere antibodies and low-titer IgM antiphospholipid antibodies, but negative for anti- β 2GP1 and anticardiolipin antibodies and lupus anticoagulant. Routine investigations excluded cancer and infection. Antibiotics, iloprost (continuous infusion), enoxaparin (100 U/kg, twice a day), and corticosteroids were administered, along with transdermal opioids for pain control. The patient received 6 courses of plasmapheresis (1 course every 3 days). After the last course, she received a 3-day intravenous pulse of methylprednisolone (1 g) followed by prednisone oral therapy (50 mg/d). This regimen did not improve the ischemic areas, and the patient progressively worsened. Limb angiography revealed bilateral occlusion of the ulnar arteries and occlusion of the interosseous arteries in the distal part of the left forearm; the palmar and digital arteries were undetectable in the medial part of the right hand. Femoral, popliteal, and anterior and posterior tibial arteries were barely visible in the distal part of the lower limbs (**Figure 2**, *A*). A vasodilator test with papaverine and nitroglycerin derivatives confirmed that the circulatory impairment was due to vascular occlusion.

Revascularization procedures were excluded, and the surgeon suggested a bilateral amputation at elbow and knee level. High-dose cyclophosphamide and hematopoietic stem-cell transplantation were both excluded because of the extent of the necrotic areas and the signs of local infection. In an attempt to recover the vascular network, we decided to infuse expanded autologous mesenchymal stem cells. Three mesenchymal stem-cell infusions were administered, 1 infusion per month, through a central venous (Groshong) catheter in the internal jugular vein. Background therapy was not changed during the procedure. Angiography was performed again 1 week after the third infusion. Appendix Figure 1 (available at www.annals.org) summarizes the time course of treatments and the timing of angiography, skin biopsies, and main outcomes at follow-up.

Context

By differentiating into endothelial cells, mesenchymal stem cells may help form new blood vessels.

Contribution

This case report describes a young woman with systemic sclerosis and acute occlusive ischemia and gangrene of the upper and lower limbs. After intravenous infusions of autologous bone marrow-derived mesenchymal stem cells, skin necrosis decreased. Postinfusion angiography showed new vessel networks in the upper limbs and distal vascularization with blood flow in the lower limbs.

Implication

Infusion of autologous mesenchymal stem cells for patients with severe ischemic peripheral vascular disease is a promising therapy. Evaluation of its benefits and harms in a larger selection of patients is now needed.

—The Editors

METHODS

The ethics committee approved the treatment, with the restriction that biopsy of the involved skin (forearms and legs) could occur only after the last mesenchymal

Figure 1. Patient's lower limbs at admission (*A*) and after receiving 3 cycles of expanded autologous bone marrow-derived mesenchymal stem cells by intravenous infusion (*B*).



Figure 2. Angiography of patient at admission (A) and after receiving 3 cycles of expanded autologous bone marrow-derived mesenchymal stem cells by intravenous infusion (B).



Arrows highlight principal areas of change, with clear blood flow in the arteries of the upper and lower limbs.

stem-cell infusion. After the patient gave informed consent, infusion was started.

Isolation, Culture, and Immunophenotyping of Mesenchymal Stem Cells

Before each procedure, bone marrow cells were harvested twice through an iliac crest aspirate (10 and 12 mL, respectively). Mesenchymal stem cells were isolated from the bone marrow buffy coat, expanded, and characterized for the expression of surface markers and differentiation properties, as described elsewhere (6, 7). The mesenchymal stem-cell product we administered was checked for sterility and *Mycoplasma* contamination by using the Mycoplasma Detection Kit (Roche Diagnostics, Mannheim, Germany). Karyotype analysis was performed by using G-banding techniques.

Mesenchymal Stem-Cell Infusion

In the first infusion, we used cryopreserved autologous mesenchymal stem cells $(0.9 \times 10^6 \text{ cells/kg})$ at first passage. Mesenchymal stem cells were thawed in a 37 °C bath, diluted with 1% saline and albumin in a 1:1 ratio to reach $1 \times 10^6 \text{ cells/mL}$, and infused intravenously through a central venous catheter. In the second and third infusions, we used second-passage mesenchymal stem cells $(0.8 \times 10^6 \text{ cells/kg})$. The cells were harvested from the culture

flask with 0.05% trypsin–ethylenediaminetetraacetic acid (Gibco, Invitrogen, Carlsbad, California), washed 3 times to remove fetal bovine serum and trypsin, and resuspended in saline supplemented with 1% human albumin (Kedrion, Lucca, Italy) at 1×10^6 cells/mL for intravenous infusion.

Skin Histopathology and Immunohistochemistry

After the third infusion, skin biopsy specimens were fixed in formalin and embedded in paraffin. Sections were processed for hematoxylin–eosin staining. Immunohistochemistry was performed on serial sections with an indirect immunoperoxidase method by using anti–human angiopoietin-1 and -2 and vascular endothelial growth factor antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Sections were observed under a light microscope (Nikon Eclipse E400, Nikon, Tokyo, Japan) and photographed by digital camera (Nikon Coolpix 2500, Nikon). Negative control images were obtained by omitting primary antibodies.

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RESULTS

Expansion and Characterization of Mesenchymal Stem Cells in Culture

The human bone marrow-derived mesenchymal stem cells displayed the typical spindle-shaped structure. Their second-passage cellular expansion capability did not differ from that of historical control samples. Testing the stem cells with the Mycoplasma Detection Kit yielded negative results. The karyotype analysis was normal. After the dead cells were excluded by using 7-aminoactinomycin D, the cell population at infusion was uniformly positive for CD29, CD44, CD166, CD90, CD73, CD105, and HLA-ABC. Antigens HLA-DP, -DQ, and -DR were expressed in 1.6% of the stem-cell population at the first infusion and in 5.9% at the second and third infusions. No contamination of hematopoietic cells was found (flow cytometry results were negative for markers of hematopoietic lineage, including CD14, CD34, and CD45). Isolated mesenchymal stem cells differentiated in osteocytes after 21 days of stimulation. Aggregates or nodules of hydroxyapatite-mineralized matrices were intensely redstained with Alizarin Red-S in both systemic sclerosis and control mesenchymal stem cells. Cells treated with adipogenic medium differentiated into adipocytes in both patient and control samples (lipid vacuoles stained orange-red after 21 days).

Patient Follow-up

We observed a reduction in necrotic skin after the first mesenchymal stem-cell infusion and a further reduction and the presence of granulation tissue and bleeding after the second and third infusions (Figure 1, *B*, and Appendix Figure 1). After the third infusion, angiography revealed a new vessel network in the upper limbs and a distal vascularization in the lower limbs, with clear blood flow in the interosseous and both tibial arteries on the right and in the interosseous and posterior tibial arteries on the left (Figure 2, *B*, and Appendix Figure 1).

Analysis of Skin Biopsy Specimens

Specimens from the forearm and leg biopsies showed reduced epidermal thickness, with scarce, flattened scales in the horny layer; keratinocytes of the lower epidermal layers still contained keratohyalin granules. Dermoepidermal papillae were flattened. In the papillary dermis of the forearm, microvessels were absent. In the leg, the papillary dermis had a regular distribution of cell clusters from the subpapillary plexus toward the dermoepidermal junction. A marked dermal hyalinosis was evident. Angiopoietin-1 was strongly expressed in clusters of cells arranged in a tubelike structure at the dermoepidermal junction. Perivascular cells in the subpapillary plexus were immunopositive for angiopoietin-1. In the papillary dermis, endothelial cells of newly formed vessels were positive for angiopoietin-2. Next to the dermoepidermal junction, vascular endothelial growth factor expression was evident in microvascular endothelial cells (**Appendix Figure 2**, available at www.annals .org).

DISCUSSION

In this patient with systemic sclerosis, our intravenous infusion of expanded, autologous, bone marrow–derived mesenchymal stem cells substantially reduced skin necrosis. The results of angiography and the strong expression of angiogenic factors in regenerating tissues also suggests that mesenchymal stem cells promoted new vessel formation and vascular remodeling in the upper and lower limbs. Angiopoietins and vascular endothelial growth factor are both involved in angiogenesis and new vessel maturation, and mesenchymal stem cells have been shown to promote angiogenesis through the upregulation of both in an experimental model of wound healing (8).

The success of mesenchymal stem-cell infusion in graft-versus-host disease has fostered a growing interest in the potential therapeutic application of mesenchymal stem cells in autoimmune diseases (9). In vitro studies and experimental models have shown that mesenchymal stem cells could be a promising therapeutic strategy for treating severe peripheral vascular disease (2, 3). In a rat model of acute kidney injury, infusing mesenchymal stem cells after severe ischemia-reperfusion episodes had a nephroprotective effect (10). Mesenchymal stem cells also improved cardiac function through angiogenesis and myogenesis in experimental models of myocardial infarction and dilated cardiomyopathy (11). Evidence indicates that mesenchymal stem cells were not incorporated into the injured vasculature, which suggests that their protective and regenerative actions might be mediated by paracrine effects (11).

Other investigators have yielded promising results with similar uses of stem-cell therapy. Ishigatsubo and colleagues (12) found a reduction in both the number and size of treatment-resistant digital ulcers and nail-bed neovascularization with intramuscular injection of autologous bone marrow-derived mononuclear cells into the ischemic limbs of patients with systemic sclerosis (12). Nevskaya and colleagues (13) applied peripheral blood-derived or bone marrow-derived CD34⁺ cells locally to digital ulcers, which resulted in accelerated healing (13). Our case differs from these cases mainly by the injection site (intravenous) and the type of cells (expanded autologous bone marrow-derived mesenchymal stem cells) used. In our case, the injected mesenchymal stem cells regenerated the vascular network and reduced skin necrosis without adverse effects, possibly because mesenchymal stem cells can differentiate into endothelial and vascular smooth-muscle cells in ischemic microenvironments (14, 15) and induce the formation of new blood vessels. Further studies on a larger number of patients with systemic sclerosis are needed

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to confirm the short- and long-term efficacy and safety of mesenchymal stem-cell infusion-based treatment of severe digital ulcers and gangrene of the extremities that are resistant to conventional therapies.

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Appendix Figure 1. Schematic representation of the time course of treatments and main outcomes at follow-up.



Despite drug administration, skin necrosis rapidly increased before MSC infusion was started. The timing of MSC infusions, angiography, and skin biopsy are shown, as well as the pharmacologic treatments that the patient continued to receive during the MSC infusions and follow-up. MSC = mesenchymal stem cell.



Appendix Figure 2. Angiopoietin-1 and -2 and vascular endothelial growth factor immunostaining of skin biopsies of the leg.

The immunoreaction was performed by an indirect immunoperoxidase method, with 3,3'-diaminobenzidine tetrahydrochloride substrate used as chromogen (brown staining). The skin sections shown in panels A to C were counterstained with hematoxylin (blue staining). A. Clusters of cells arranged in a tubelike structure (*arrows*) show angiopoietin-1 expression at the dermoepidermal junction. Perivascular cells show angiopoietin-1 immunopositivity in the subpapillary plexus (stain; original magnification, $\times 20$). B. Higher magnification view of panel A (stain; original magnification, $\times 40$). C. In the papillary dermis, endothelial cells of newly formed vessels (*arrows*) express angiopoietin-2 (stain; original magnification, $\times 20$). D. Endothelial cells of microvessels (*arrow*) display strong positivity for vascular endothelial growth factor in the papillary dermis (stain; original magnification, $\times 25$). Inset. Higher magnification view that shows vascular endothelial growth factor-positive microvascular endothelial cells (stain; original magnification, $\times 60$).