Regulatory T cells enhance mesenchymal stem cell survival and proliferation following autologous cotransplantation in ischemic myocardium

Yifu Zhou, MD, Avneesh K. Singh, PhD, Robert F. Hoyt, Jr, DVM, Suna Wang, MD, Zuxi Yu, MD, PhD, Timothy Hunt, BS, Bogdan Kindzelski, BS, Philip C. Corcoran, MD, Muhammad M. Mohiuddin, MD, and Keith A. Horvath, MD

Objectives: We sought to investigate if autologous freshly isolated regulatory T cells (Tregs) provide a protective and supportive role when cotransplanted with mesenchymal stem cells (MSCs).

Methods: In a porcine model of chronic ischemia, autologous MSCs were isolated and expanded ex vivo for 4 weeks. Autologous Treg cells were freshly isolated from 100 mL peripheral blood and purified by fluorescence-activated cell sorting. MSCs and Treg cells were then cotransplanted into the chronic ischemic myocardium of Yorkshire pigs by direct intramyocardial injection (1.2×10^8 MSCs plus an average of 1.5 million Treg cells in 25 injection sites). Animals were killed 6 weeks postinjection to study the fate of the cells and compare the effect of combined MSCs + Treg cells transplantation versus MSCs alone.

Results: The coinjection of MSCs along with Tregs was safe and no deleterious side effects were observed. Six weeks after injection of the cell combination, spherical MSCs clusters with thin layer capsules were found in the injected areas. In animals treated with MSCs only, the MSC clusters were less organized and not encapsulated. Immunofluorescent staining showed CD25+ cells among the CD90+ (MSC marker) cells, suggesting that the injected Treg cells remained present locally, and survived. Factor VIII+ cells were also prevalent suggesting new angiogenesis. We found no evidence that coinjections were associated with the generation of cardiac myocytes.

Conclusions: The cotransplantation of Treg cells with MSCs dramatically increased the MSC survival rate, proliferation, and augmented their role in angiogenesis, which suggests a new way for future clinical application of cell-based therapy. (J Thorac Cardiovasc Surg 2014;148:1131-7)

Cell-based therapy using either mesenchymal stem cells (MSCs) or induced pluripotent stem cells has been broadly used in animal models of myocardial ischemia or infarction to improve heart function or to regenerate damaged myocytes.[1-6] We previously reported that autologous transplantation of MSCs led to improvement in global left ventricular function and regional wall thickening in an ischemic myocardium.[7] However the issue of cell survival after transplantation is still a major obstacle for cell-based therapy. Efforts have been focused on stem cell gene manipulation[8,9] or using materials such as hydrogel[10] that would help to increase cell survival and homing following transplantation. These efforts have been shown to be of reliable benefit in animal models; however, when used clinically, potential risks or side effects cannot be excluded following gene manipulation or the use of adjunct materials for improved stem cell survival. Studies have shown that $CD4^+CD25^{hi}FoxP3^+$ T regulatory (Treg) cells have the potential to suppress inflammation, promote angiogenesis, induce tolerance, and provide a favorable environment for cellular engraftment.[11,12] We sought to investigate if autologous Treg cells provide a protective and supportive role when cotransplanted with MSCs in an animal model of chronic ischemia.

MATERIAL AND METHODS

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, and the investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996, Washington, DC). Yorkshire domestic pigs, initially weighing 15 to 20 kg, were selected for this study. All animals were housed 1 per cage and allowed free access to water and commercial pig food.

Study Design

Fifteen animals underwent a small left thoracotomy under general anesthesia and had placement of an ameroid constrictor around the

From the Cardiothoracic Surgery Research Program, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md.

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Address for reprints: Yifu Zhou, MD, Cellular Biology Section, CSRP/NHLBI, National Institutes of Health, 10 Center Dr, MSC 1550, Bldg 10, Rm B1-D47, Bethesda, MD 20892-1550 (E-mail: zhouyifu@mail.nih.gov).

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Abbreviations and Acronyms

G-CSF = granulocyte colony-stimulating factor
LCX = left circumflex artery
MSC = mesenchymal stem cell
PBMC = peripheral blood mononuclear cells
PBS = phosphate-buffered saline
Treg = regulatory T cell

proximal left circumflex artery (LCX) to create a model of chronic myocardial ischemia. At this first operation, bone marrow (about 15 mL) was harvested for ex vivo stem cell expansion. Four weeks later, a second left thoracotomy was performed in each animal. The circumflex territory (ischemic zone) was exposed and injected with ex vivo expanded MSCs that were mixed with freshly isolated Tregs in 7 animals. Six animals received only MSCs as control. Six weeks following cell injection, all animals were killed, and the hearts were harvested for histopathologic evaluation (Figure 1).

Chronic Ischemia Model

All animals were anesthetized and underwent a left-sided thoracotomy. The pericardial sac was partially opened to facilitate dissection and visualization of the LCX as it branches from the left coronary artery. After LCX exposure, a 2.5- to 3.5-mm titanium-encased ameroid was placed around the proximal LCX. The pericardial sac was then closed to minimize adhesion formation. The ameroid constrictor gradually occludes the LCX over a period of 3 to 4 weeks resulting in a region of myocardial ischemia of the left ventricle.[13] Fifteen to 20 mL bone marrow was aspirated during the ameroid placement procedure while the animals were still under general anesthesia. To help prevent arrhythmias, all animals were given amiodarone preoperatively beginning 5 to 7 days before the second surgery, which was continued until harvest.

Preparation and Culture of Bone Marrow–Derived Cells

Using aseptic technique, bone marrow was aspirated from either the iliac crest or tibia of the pig into a syringe containing preservative-free heparin. Peripheral red blood cells were separated by gradient centrifugation using lymphocyte separation liquid. The middle layered cells were collected and divided into 2 populations: 1 population was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1× penicillin-streptomycin in a density of $10^6/cm^2$ at 37°C with 5% carbon dioxide in T-75 culture flasks without coating. When the attached cells reached confluence, they were split and expanded for 3 to 4 passages. Cells were assessed for cell surface markers, multipotency of differentiation, and karyotype analysis.

Isolation and Purification of Tregs

Fresh autologous Tregs were isolated and purified the same day that the animals were scheduled for MSC injections. Peripheral blood (100 mL) obtained from each animal with chronic left myocardial ischemia was diluted at a ratio of 1:2 with phosphate buffered saline (PBS) and centrifuged over a ficoll-hypaque gradient to obtain peripheral blood mononuclear cells (PBMCs). The remaining red blood cells in the PBMC solution were lysed by 1× PharmLyse (BD Biosciences, Mountainview, Calif) according to the manufacturer's instructions and then washed twice with PBS. The isolated PBMCs from each animal were then incubated with the mouse anti pig CD4 (clone 74-12-4; BD Biosciences) and mouse anti-pig CD25 (clone K231.3B2; GeneTex; Irvine, Calif) antibodies for 30 minutes. After the incubation, PBMCs were washed 2 times with PBS and then incubated with secondary antibody-goat antimouse anti-immunoglobulin G1conjugated with phycoerythrin (SBA, Birmingham, Ala) for 30 minutes and washed 2 times with PBS. The Treg cells (CD4+CD25+) were then purified by flow cytometry cell sorting using FACS Aria (BD Biosciences) cell sorters. Dead cell exclusion was done with 7-aminoactinomycin D.

Treatment of Ischemic Zone With Bone Marrow–Derived MSCs Plus Peripheral Blood-Derived Tregs

Four weeks following ameroid placement, ex vivo expanded autologous MSCs (1.2×10^7) were mixed with freshly isolated and purified peripheral blood derived Tregs (average of 1.5×10^6) in 2.5 mL saline. This cell mixture was injected intramyocardially in each animal with a 25-gauge needle (with an injection depth of 0.5 mm) around the left myocardial ischemic zone (25 injection sites, 100 μL in each site). As controls, 6 animals underwent same ameroid placement and bone marrow extraction procedure, but received MSCs only.

Histologic and Immunohistochemistry Analysis

After recipient pig euthanasia, transmyocardial tissue clumps of the suture marked injection area within ischemic (LCX territory) myocardium, ischemic area with no injection, or nonischemic (right ventricle territory) myocardium were cut into 5 × 5 mm-thick pieces. These tissue sections were collected in cassettes and fixed with 10% buffered formalin for paraffin embedding or embedded in optimal cutting temperature medium for frozen sections with no fixation. The tissue clumps from the injected areas were further cut into 4 pieces and collected separately to track the injected cells. Paraffin-embedded sections were stained with hematoxylin and eosin and Masson trichrome for morphologic analysis. Immunohistochemical or immunofluorescent staining was performed using the mouse monoclonal antihuman antibodies (which cross-react with pig) to CD90, (BD Biosciences), and mouse anti-pig CD25. The incubations of the primary antibodies were followed by detections of fluorescein isothiocyanate or rhodamine conjugated with antimouse immunoglobulin G and the nuclei were labeled with 4′, 6-diamidino-2-phenylindole.

RESULTS

Determination of Morphology and Surface Expression of MSCs and Tregs

Bone marrow–derived MSCs showed uniformed fibroblast-like morphology. After 4 weeks of ex vivo expansion, the total number of cells reached 1.2×10^8. Fluorescence-activated cell sorting analysis at passage 4 revealed cells strongly positive for cell surface expression of CD44 and CD90, but negative for CXCR4, CD34, C-kit, CD144, CD54, CD45, and CD31 surface markers. These results indicate that bone marrow–derived stem cells are MSCs, instead of endothelial progenitor cells. Positive adipogenesis and osteogenesis testing confirmed the multipotency of these cells. The normal karyotype pattern ensured the MSCs were not transformed over the 4 weeks of ex vivo expansion. The purity of peripheral blood derived freshly isolated autologous Tregs was >95% after cell sorting (Figure 2).

Animal Survival

In our preliminary study, 2 animals died of heart failure following ameroid placement before cell treatment,