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

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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 hydrogel¹⁰ 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

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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 $10^6/\text{cm}^2$ 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, Mountain-view, 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 G1 conjugated 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,

FIGURE 1. Diagram showing the experimental timeline from ameroid placement, mesenchymal stem cells and regulatory T cells injection, to the end of the experiment. *MSC*, Mesenchymal stem cell; *LCX*, left circumflex artery; *LAD*, left anterior descending artery.

possibly due to myocardial infarctions or arrhythmias. One animal in the MSC plus Treg group died after cell injection due to a surgical wound infection. Six animals survived to the scheduled euthanasia in both groups. All animals following MSCs plus Tregs, or MSCs-only injection showed no signs of severe side effects related to cell injection.

Histologic Analysis

Six weeks after the combined cell therapy (MSCs plus Tregs), no observable fibrosis was found on histopathology. Under light microscopy, spherical MSC clusters with thin layers (~15-20 μ m in thickness) of capsules were found in the injected areas in all six treated animals (Figure 3). These cell clusters can be found in all sections (with 2- to 6-in each section) under low power (12.5 $\times$) magnification. In contrast to animals treated with Treg and MSC injections, animals that received only MSC injections had cell clusters in all sections with similar numbers in each section;

however, these MSC clusters were less organized and not encapsulated (Figure 4). This finding may suggest that with Treg cotransplantation, MSC survival was increased. Additionally, MSCs may also proliferate locally in the presence of Tregs. Immunofluorescent staining showed that the CD25+ cells were found among the CD90+ cells, suggesting that even after 6 weeks of injection, Treg cells remained present locally, and survived (Figure 5). No MSC clusters were found in the noninjected ischemic or normal myocardial area. The CD25+ cells were also not detectable in the MSC only sections. Factor VIII and smooth muscle actin positive cells were also prevalent around the cell cluster area suggesting the formation of new vessels (Figure 6). We found no evidence that coinjections were associated with the generation of new cardiac myocytes.

DISCUSSION

Clinical application of stem cell therapy in cardiovascular disease has been used during the past decade in several

FIGURE 2. Freshly isolated pig natural regulatory T cells were purified with flow cytometry cell sorting. *Left*, Gated lymphocytes. *Center*, CD4+ and CD25+ cells before sorting. *Right*, Both CD4+ and CD25+ regulatory T cells after sorting and ready for cotransplantation with mesenchymal stem cells. *SSC*, Side-scattered light; *FSC*, forward-scattered light.

FIGURE 3. Hematoxylin and eosin staining of a representative animal at 6 weeks after mesenchymal stem cells plus regulatory T cells injection showing spherical cell clusters with thin layers of capsules in frozen and paraffin-embedded sections. A, Arrow indicates the capsule around the cell clump ($\times 100$) in a frozen section. B, Higher magnification of (A) ($\times 400$). C, Three cell clusters were found inside myocardium ($\times 100$) in a paraffin-embedded section. D, Higher magnification of (C) ($\times 200$).

different ways: through the route of intracoronary injection using balloon/catheter devices, intravenous injection, and/or using granulocyte colony-stimulating factor (G-CSF) to stimulate the mobilization of bone marrow cells

into the circulation.¹⁴⁻²⁴ Beneficial effects or functional improvements have been reported using these cell-based therapies. However it is obvious that large portions of intracoronary injected cells flow into the bloodstream and

FIGURE 4. Hematoxylin and eosin staining of a representative animal at 6 weeks after mesenchymal stem cells injection without regulatory T cells (paraffin-embedded sections). A, Cell clusters were found in the border area of epi- and myocardium ($\times 100$). B, Higher magnification of (A) ($\times 200$). C, A cell cluster was found inside myocardium ($\times 100$). D, Higher magnification of (C) ($\times 200$). Arrows indicate the cell clusters.

FIGURE 5. Immunofluorescent staining of CD25 (green), CD90 (red), and 6-diamidino-2-phenylindole (blue) in frozen section at 6 weeks after mesenchymal stem cells plus regulatory T cells injection. A, CD25+ (regulatory T cells marker) cells were found inside the cell cluster with CD90+ (mesenchymal stem cell marker) cells ($\times 200$). B, Higher magnification of (A) ($\times 400$). Arrows indicate CD90+ cells and arrowheads indicate CD25+ cells. C, Stained with no first antibodies (control). D, Higher magnification of (C).

only a small number of the cells actually migrate into and remain in the ischemic myocardium. The majority of nonexpanded mononuclear cells from bone marrow are

hematopoietic lineage cells, so that the beneficial effects created by these cells are limited. The intracoronary injection is only applicable for nonex vivo expanded cells

FIGURE 6. Immunofluorescent staining of Factor VIII (green), smooth muscle actin (red), and 6-diamidino-2-phenylindole (blue) in frozen section at 6 weeks after mesenchymal stem cells plus regulatory T cells injection. A, Increased capillary density suggested by Factor VIII+ and smooth muscle actin staining around the spherical cell cluster compared with (B) (no cluster area). C, A typical spherical cell cluster from another animal. D, Negative control (no antibodies were used). Arrows indicate cell clusters. Magnifications are $\times 100$.

(or after short expansion times of 4 days or fewer), because the cell size of expanded cells are much bigger than nonexpanded cells (10–15 μm vs 5 μm in diameter). G-CSF mobilized cells are also heterogeneous, only a small fraction is endothelial progenitors and may or may not contain any MSCs. Therefore the above 2 methods of using nonexpanded bone marrow–derived cells or G-CSF mobilized cells will dramatically limit their benefits. We recently used direct injection of expanded MSCs into the ischemic area to facilitate the ability to determine both the beneficial effects and the fate of the injected cells.⁷ Because this method entails direct injection to target tissues, there are no issues related to homing. Unfortunately, this is not a perfect technique either, because the amount of MSCs is also reduced dramatically following injection. This loss in cell number occurs in 2 ways: loss immediately after injection due to continuous myocardial contraction and gradual loss from the treated area over the course of follow up due to a limited self-renewing rate.²⁵ Therefore, several ways have been used to change the behavior of the stem cells by gene manipulation or using specific biocompatible materials (eg, hydrogel) to prevent the first wave loss after injection. However, the use of these methods in patients poses potential risks and side effects.

Recent reports on the interactions between MSCs and Tregs appear favorable and may mitigate the loss of MSCs after treatment.^{11,12} Tregs are a specialized subset of T cells that facilitate self-tolerance and are known to express both CD4 and CD25.²⁶ The depletion of these Tregs will spontaneously evoke autoimmune disease in otherwise normal rodents, suggesting their major role in suppressing the unfavorable immune response.²⁶ Recently Tregs have been reportedly related to tumor hypoxia, promoted tolerance, and angiogenesis.¹² Additionally, MSCs have been found to interact with Tregs through interleukin 10, prostaglandin E2, and transforming growth factor β , or by cell-to-cell contact mechanisms.²⁷ We sought to study the interactions between the MSCs and Tregs once they were cotransplanted in the ischemic myocardium. Our results demonstrate that Tregs dramatically changed the behavior of MSCs in vivo—the self-renewing rate was increased evidenced by the shape of the cell clumps and the layers of capsules, which usually characterize the fast growing cells. Even though we cannot be certain of the degree of benefit that the Tregs helped to prevent the early loss of MSCs, it is clear that the Tregs aided in MSC survival and self-renewal, as a result of their possible paracrine effects. Because the Tregs we employed were autologous, there were no immune rejection issues or other host versus donor disease processes. In this study, we did not find evidence showing the Tregs within the myocardial environment caused any detrimental side effects. One limitation of using Tregs is their very low number in peripheral blood. To obtain Tregs in large numbers the naïve

Tregs need to be expanded. Several protocols are available for this purpose. Availability of a large number of Tregs will allow better understanding of their role in improving MSC function in myocardial recovery and regeneration.

Dhingra and colleagues²⁸ recently reported that MSCs stimulate the proliferation of Tregs through interaction with CCL5 and CCL12. Tregs also increase the cell survival of both allogeneic and syngeneic cells implanted into the ischemic myocardium. Therefore, it is possible that CD25+ cells surrounding the CD90+ cells in the heart may be partially due to the endogenously recruited Tregs. Furthermore, the combined group may have improved the metabolic activity of the injected MSCs so that they stimulated the production of more endogenous Tregs rather than the survival of the injected Tregs. However in our study no MSC clusters were found in the noninjected ischemic or normal myocardial area. CD25+ cells were also not found in the MSC-only sections. Therefore, our results suggest that Treg and MSC interactions help both cell subsets' survival after injection and possible recruitment of these cells.

Tregs may play very important roles in the inhibition of allogeneic reaction against transplanted MSCs.²⁹ Thus, using Tregs in allogeneic MSC transplantation needs to be explored. Our study provides preliminary data within this field. Functional improvement and cell-to-cell interaction in vitro studies are still needed to confirm the true beneficial effects of Tregs and to define their underlying mechanisms of action.

SUMMARY

This is the first in vivo study of autologous Treg and MSC transplantation in a large animal model of myocardial ischemia. We have shown here that autologous injection of bone marrow–derived MSCs with freshly isolated and purified Tregs is safe. No observable side effects or abnormal cell proliferation was found in the cotransplantation study. This method of cotransplanting Tregs with MSCs showed a beneficial effect on MSC survival and self-renewing. The results from our study will help with future clinical applications of cell-based therapy.

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Discussion

Dr Richard Weisel (Toronto, Ontario, Canada). Thank you very much for an excellent presentation and congratulations to your group for a very difficult study in these large animal models. This study represents a real tour de force. The proposal to enhance mesenchymal stem cell (MSC) engraftment is very important. Another approach is to use the hydrogels that were described earlier to improve cell survival. Another alternative is to reduce the inflammatory phase, as Dr Mayer discussed earlier, which is a very important concept. Of course this is what MSCs do normally. The implantation of MSCs into the heart induces the proliferation of T regulatory cells (Tregs), 1 of the major beneficial effects of MSC treatment. Therefore, the transplantation of Tregs with MSCs is enhancing the endogenous repair mechanisms that result from MSC therapy. This approach is a great idea.

Did you actually improve MSC survival? Was the number of MSCs in the heart greater with the Tregs?

Dr Zhou. These are good questions. It is difficult to quantitatively count the total number of these cells at 6 weeks after injection to see if they increased. However, evidence of an increase includes the very dense and sphere-shaped clusters surrounded by thin, layered capsules seen on serial tissue section analysis. These were never found in the MSC-only injection heart. We believe the cell number was increased compared with MSC only, but it's difficult to give a quantitative answer to this question.

Dr Weisel. Did you improve heart function?

Dr Zhou. This is preliminary data and this project is still ongoing. We are testing the functional improvement by performing stress echo and magnetic resonance imaging before and after cell transplantation. The data will be shown in the near future.

Dr Weisel. The cotransplantation of Tregs and MSC was beneficial for autologous cell therapy but may be very important for allogeneic cell therapy. As you know, many investigators are now proposing allogeneic rather than autologous cell therapy because of the tremendous opportunity to have an off-the-shelf product for patients who need it, particularly in the operating room. Have you tried this approach for allogeneic cells? Cotransplantation of Tregs and MSCs would be much more relevant for allogeneic cell therapy.

Dr Zhou. We tried allogeneic cell therapy using green fluorescent cells and published those results previously. But during that study we did not involve Tregs. I think it is an excellent idea to do it in the future.