Transcoronary implantation of bone marrow stromal cells ameliorates cardiac function after myocardial infarction

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Objectives: Bone marrow stromal cells are capable of differentiating into cardiomyogenic cells. We tested the hypothesis that transcoronary implantation of bone marrow stromal cells may regenerate infarcted myocardium and reduce cardiac dysfunction.

Methods: Isolated bone marrow stromal cells from the isogenic donor rats were transfected with LacZ reporter gene for cell labeling. To induce cardiomyogenic differentiation, the bone marrow stromal cells were treated with 5-azacytidine before implantation. Two weeks after left coronary ligation, these cells (1×10^6 in 150 μ L) were infused into the briefly distally occluded ascending aorta of the recipient rats ($n = 15$) to simulate direct coronary infusion clinically. Control animals were infused with cell-free medium ($n = 14$). Cardiac function was evaluated by echocardiography at preimplantation and 4 and 8 weeks postimplantation. The hearts were then immunohistochemically studied to identify phenotypic changes of implanted bone marrow stromal cells.

Results: Immediately after cell infusion, the bone marrow stromal cells were trapped within coronary vessels in both infarcted and noninfarcted areas. However, after 8 weeks, most of the cells were identified in the scar and periscar tissue, expressing sarcomeric myosin heavy chain and cardiomyocyte-specific protein troponin I-C. Some bone marrow stromal cells were found to be connected to the adjacent host cardiomyocytes with gap junction. Two-way repeated-measures analysis of variance revealed significant improvement in fractional shortening and end-diastolic and end-systolic diameter of the left ventricle ($P = .0465, .002, .0004$, respectively) in the bone marrow stromal cell group.

Conclusions: Although bone marrow stromal cells had been reported to improve cardiac function when injected directly into the myocardial scar, this study demonstrated for the first time that bone marrow stromal cells can be delivered via the coronary artery, as they are capable of targeted migration and differentiation into cardiomyocytes in the scar tissue to improve cardiac function.

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With the recent advent in stem cell biology, it has been shown that an adherent population of bone marrow cells in culture that can be expanded in vitro, known as “marrow stromal cells” (MSCs), contain adult stem cells that can give rise to various mesenchymal and nonmesenchymal cell types.¹⁻⁴ Since Makino and colleague⁵ demonstrated that cardiomyogenic differentiation of MSCs in vitro in 1999, several in vivo studies including

those of ours have confirmed this capability of the MSCs.⁶⁻⁹ The advantages of using MSCs as donor cells for cellular cardiomyoplasty are that MSCs can be easily harvested from patients' own bone marrow by aspiration, can be expanded vastly in culture to provide adequate numbers, and can then be autoimplanted without encountering immunorejection. A clinical trial for the autologous transplantation of bone marrow cells into an unrevascularizable area of the ischemic hearts to induce angiogenesis has already been reported.¹⁰

Cells can be implanted into the myocardium in a number of ways. To date, most of the experimental studies reported have employed direct injection of various types of donor cells into the myocardium.^{6,7,9,11-14} The advantage of this procedure is that it enables one to transplant cells selectively into specific areas of the myocardium. However, such an approach is rather invasive and in particular not suitable for patients with diffuse cardiomyopathy. In a recent study, we confirmed the feasibility of delivering MSCs via the coronary artery.⁸ As the MSCs were found to be capable of migrating to an injured area after transplantation,^{15,16} we hypothesized that MSCs, when infused into the coronary artery, could engraft into an infarcted myocardium and participate in myocardial regeneration and perhaps also angiogenesis,¹⁰ resulting in an improved cardiac function. In this study, we used a rodent model proximally injecting MSCs into the briefly occluded ascending aorta, to simulate clinical coronary delivery of these cells such as by selective coronary artery catheterization, or by aortic root injection during cardiopulmonary bypass.

Materials and Methods

Animals

Male inbred Lewis rats (200 to 250 g) were obtained from Charles River Laboratory (Laprairie Co, Quebec, Canada). These isogenic rats were used as donors and recipients to simulate the autologous implantation of MSCs. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Academy Press (revised 1996), and the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animals Care.

Isolation and Cell Labeling

Isolation and primary culture of MSCs from the femoral and tibial bone of donor rats were performed as previously described in detail.^{7,8} A replication defective retrovirus containing the reporter (LacZ) gene that encodes for the bacterial β -galactosidase enzyme were produced by LacZ-GP+AM12 amphotropic retrovirus producer cells originally obtained from Dr Jacques Galipeau's Laboratory (McGill University, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada).¹⁷ The second-passaged MSC growth medium was replaced with the supernatant from the LacZ-GP+AM12 cells as described in detail before.⁸

MSC Treatment with 5-aza-2'-deoxycytidine (5-azacytidine)

MSCs that were harvested and labeled were treated for 24 hours with 5-azacytidine [0.3 μ M solution in 10% Dulbecco's modified Eagle's medium (DMEM)] within the culture dish. The cells were incubated overnight in a 37°C incubator with a humidified atmosphere of 5% CO₂. The following day, the supernatant was removed and replaced with 10% DMEM solution for 24 hours. The cells were treated once more with 5-azacytidine for 24 hours and the growth medium replaced. MSCs treated with 5-azacytidine were infused into the coronary artery of recipient animals 24 hours after the treatment.

Myocardial Infarction and Infusion of MSCs

Forty-one rats underwent ligation of the left coronary artery. Rats were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and intubated and mechanically ventilated at 80 breaths/min. The heart was exposed via a left thoracotomy incision. The left coronary artery was ligated proximally with a 6-0 polypropylene suture.⁸ Two weeks after the coronary ligation, survived rats (n = 29) were divided randomly into two groups; rats infused with MSCs (MSC group: n = 15) and rats infused with culture medium (control group: n = 14). Anesthesia was induced and maintained with isoflurane. The ascending aorta was exposed through upper median sternotomy and looped with a suture after dissection. Under direct vision, transfected MSCs suspension (1×10^6 cells in 150 μ L of DMEM) was then injected into the root of the ascending aorta, while distally occluding the aorta briefly for less than 3 seconds using the looped suture. A 28-G needle on an insulin syringe was used for the cell injection.⁸ Equivalent volume of serum-free culture medium was injected into the control animals using the same procedure. Two animals from the MSC group were sacrificed at 10 minutes after the injection to observe initial cell distribution.

Echocardiography

Transthoracic echocardiography was performed on all animals at 2 weeks after coronary ligation before infusion of MSCs as baseline and at 4 and 8 weeks after injection of MSCs or cell-free medium. Echocardiographies were performed with a commercially available echocardiography system (SONO 5500, Hewlett-Packard) equipped with 7.5-MHz transducer (Hewlett-Packard), and interpreted in a blinded fashion. Under anesthesia the heart was first imaged in the 2-dimensional mode in the parasternal long-axis view of the left ventricle (LV). Measurements of maximal LV long-axis lengths (L) and endocardial area tracings (a), using the leading edge method, were carried out.¹⁸ LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were calculated by the single-plane area-length method¹⁹: $V = 8 \times a^2 / (3 \times \pi \times L)$. LV ejection fraction (EF) was then inferred: $EF = [(LVEDV - LVESV) / LVEDV]$. M-mode images were obtained at the level of papillary muscles of the mitral valves. End-diastolic and end-systolic diameter of the LV (LVEDd and LVESd) were determined. Fractional shortening (FS) was then calculated as $[(LVEDd - LVESd) / LVEDd]$.

Figure 1. β -gal activity of MSCs. Histochemical staining for β -gal activity of rat MSCs in culture. The transfected MSCs showed clear staining for β -gal activity in their nuclei. Transfection efficiency of the MSCs was approximately 100%. Original magnification: $\times 100$.

MSCs Staining With X-gal for Detection of β -galactosidase Activity

Cells were plated in 35-mm dish. They were fixed in 1% glutaraldehyde for 5 minutes at room temperature, then washed with phosphate buffered saline (PBS). Staining solution (500 μ L) at pH of 8.0 was added, which contained 1 mg/mL 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal), 1 mmol/L ethyleneglycol-bis-(β aminoethylether)-N,N',N'-tetraacetic acid, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mmol/L magnesium chloride, and 0.01% sodium deoxycholate.⁸ Then cells were incubated at 37°C while protected from light for 16 hours.

Tissue Processing and Staining for β -galactosidase Activity

Animals were sacrificed at 1 day after the final echocardiogram (8 weeks after transplantation). As mentioned above, two animals in the MSC group were sacrificed immediately after cell transplantation to confirm the initial distribution of MSCs. Upon sacrifice of animals, hearts were excised. The hearts were rinsed with PBS and then perfusion fixed in 2% paraformaldehyde in PBS. The staining for β -galactosidase activity was performed as described above, but with the addition of 0.02% of Nonidet P-40 and 0.01% of deoxycholate to the staining solution.⁸ After X-gal staining at pH of 8.0, the hearts were cut longitudinally and embedded in paraffin.

Histology and Immunohistochemistry

Heart sections 5 μ m in thickness were processed for H&E, Picrosirius Red, and immunohistochemical stains. Immunohistochemical stains were done for anti- α smooth muscle actin (Sigma Laboratories), endothelial cell marker factor VIII, and cardiomyocyte specific troponin I-C (Santa-Cruz Biotechnology Inc). Stainings were also carried out for sarcomeric myosin heavy-chain molecules with MF 20 (Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child

Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences) and connexin 43 (Zymed Laboratories Inc) as previously described.⁸ Briefly, after deparaffinization, sections were placed in boiled citrate buffer (pH 6.0). After blocking in normal serum, sections were treated with the respective monoclonal antibodies overnight and with secondary antibodies the following day. Diaminobenzidine (DAB) was then used as a chromogen for light microscopy. Counterstaining of sections with hematoxylin and eosin was also performed. Cells derived from MSCs were identified by their blue nuclei (ie, β -gal labeled) with an Olympus microscopy (BX-FLA, Olympus).

Estimation of the Number of β -gal-Positive Cells in the Heart

We estimated the number of β -gal-positive cells present in the heart immediately after infusion by means of Weiss' method,²⁰ which had been validated for the rodent heart model when the cell distribution could be assumed to be homogeneous. Briefly, cell counts were made on 5- μ m-thick histological sections and surface area of the myocardium on the section was measured by planimetry. Then cell number in certain tissue volume was referred to the gross myocardium volume, which had been obtained by water displacement technique.

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Repeated variables were analyzed using a two-way repeated-measures analysis of variance. Differences at each time point were compared by unpaired *t* test. Statistical analysis was performed using a commercial software (StatView version 5.0.1, SAS Institute Inc). A *P* value of .05 or less was considered statistically significant.

Results

MSCs in Culture

The MSCs in culture were spindle-shaped, attached to the bottom of culture dish tightly, and proliferated in the culture medium. Approximately 3 to 4 passages after first culturing the cells, they were expanded to over 25 million cells from the initial 250 to 500 cells. To trace the fate of MSCs after transplantation, we labeled MSCs with replication defective retrovirus carrying the LacZ reporter gene, as described above. β -gal staining in vitro demonstrated that transfection efficiency was nearly 100%. After treatment with 5-azacytidine, the cell cultures were observed for morphological changes. However, we were not able to find myotube formation or pulsating cells by the time of implantation, as previously reported by Makino and colleagues⁵ (Figure 1).

Mortality

Mortality within the first 2 weeks after coronary artery ligation was 29.3% (12/41). Mortality within 8 weeks after randomization and coronary infusions was 13.3% (2/15) in the MSC group and 14% (2/14) in the control group.

Figure 2. Cell distribution in the myocardium. β -gal positive cells were trapped in capillaries of both noninfarcted (A and B) and infarcted myocardium (D). The hearts were harvested immediately after cell infusion. (A, B, and D) Stained for H&E; (C) immunostained for endothelial cell marker, factor VIII. Arrows indicate β -gal positive cells. Arrowheads indicate epicardium of the heart. Original magnification: $\times 40$ (A), $\times 200$ (B and D), $\times 400$ (C).

Histological and Immunological Assessment of Engrafted MSCs

Immediately after infusion of MSCs, β -gal-positive cells were consistently found in all sections of the heart. They were trapped within the coronary vasculatures in the non-infarcted (Figure 2, A to C) as well as the infarcted areas (Figure 2, D), mostly within capillaries and some in small arterioles. No MSCs could be seen in the interstitium of the scarred myocardium at this time. Approximately 3.45×10^5 MSCs, that is 34.5% of the cells injected into the ascending aorta, were estimated to have been trapped within coronary capillaries immediately after injection. Among them 73% were seen in the noninfarcted myocardium of the hearts. Most of the MSCs found in the myocardium were located in the outer layer (subepicardial) rather than the inner layer of the ventricular wall.

In contrast, nearly all of MSCs in the heart obtained at 8 weeks after cell infusion were seen within the infarct zone and scattered diffusely (Figure 3, A and B). Immunostaining for endothelial cell marker factor VIII showed most of labeled cells were located outside of the vascular lumens. These findings were consistent in all hearts of the MSC group. The cells in the periscar area appeared morphologically distinct from the cells in the middle of the scar. MSCs in the periscar area were elongated and had centrally located nuclei, with organized contractile proteins such as sarcomeric myosin heavy chain and cardiomyocyte-specific troponin I-C in their cytoplasm (Figure 4, A and B). Picrosirius Red stain of the heart section also revealed that those morphologically mature MSC-derived cardiomyocytes were mostly located in the subepicardial area of the periscar tissue (Figure 5, A and

Figure 3. Cell migration to the infarct site in the heart. Harvested at 8 weeks after cell infusion, numerous β -gal positive cells were identified in the infarcted myocardium (A and B). Arrow indicates epicardium. Original magnification: $\times 40$ (A), $\times 200$ (B).

B). On the other hand, MSCs within the scar showed less mature appearance despite positive stains for cardiac specific proteins (Figure 4, C and D). They have a larger nucleus-to-cytoplasm ratio and no recognizable striation in their cytoplasm. Some MSC-derived cardiomyocytes in the periscar area are seen to be connected to the adjacent host cardiomyocytes with positive stain for connexin 43 (Figure 4, E), a major constituent protein of gap junctions in the intercalated disks of cardiac myofibers. Some MSCs in the scar are integrated into vascular walls and stained positively for α -smooth muscle actin (Figure 4, F) or for endothelial cell marker, factor VIII.

In Vivo Cardiac Function

There was no statistically significant difference in EF ($P = .640$), FS ($P = .858$), LVEDd ($P = .122$), and LVEDs ($P = .176$) at the baseline between the MSC and control groups (Figure 6). However EF, which was obtained from long-axis view of the LV, was significantly higher in the MSC group compared with the control group following cell infusion ($P = .029$ at 4 weeks, $P = .026$ at 8 weeks). Similarly, FS, LVEDd, and LVEDs obtained from short-axis view of the LV were significantly different between the groups at 8 weeks after cell infusion (FS, $P = .025$; LVEDd, $P = .039$; LVEDs, $P = .013$). Time course changes between groups after cell or culture medium injections are significantly different in FS ($P = .0465$), LVEDd ($P = .002$), and LVEDs ($P = .0004$). However, change in EF over time did not reach statistical significance ($P = .3575$).

Discussion

In this study, we transplanted MSCs into the heart with myocardial infarction by injecting cells into briefly distally

occluded ascending aorta. We previously reported that only 2% of cells delivered could be detected in the whole heart harvested immediately after such an infusion.⁸ In the current study, however, approximately 34% of cells were identified. What we modified in cell infusion technique was to shorten aortic occlusion period (from 30 seconds down to only 3 seconds), which can minimize bleeding and leakage of donor cells from the needle-puncture site, and to give bolus injection rather than a slower infusion. After bolus injection, the cells were disseminated and trapped in vessels and capillaries of not only the noninfarcted but also the infarcted segment of myocardium. Although left coronary artery was ligated proximally 2 weeks before cell infusion, heterogeneously formed, scarred myocardium contained patent vessels even in the center of the scar, where MSCs could have reached through the collaterals. Suzuki and coworkers²¹ have transplanted skeletal myoblasts via the coronary circulation of temporarily arrested heart by cardioplegic solution using isolated perfusion apparatus, which was followed by implantation of the heart to the abdomen of recipients. In their study, engrafted cells were identified in all layers of the heart. In our study; however, more cells were found in the subepicardial zone of the myocardium rather than in the subendocardium. This could be related to the lower systolic intramyocardial pressure of the beating heart in the outer layer of the myocardium. In contrast, at 8 weeks after cell implantation, most of the cells were located in the infarcted and peri-infarcted myocardium. Individual cells were seen outside of the blood vessels and were scattered in and near the scar. These findings were consistent with our previous finding in which we reported the translocation of entrapped cells from the coronary capillaries to the myocardial interstitium.⁸

Figure 4. Phenotypic changes of the MSCs. Immunostain for troponin I-C (A and C), MF20 (B and D), connexin 43 (E), and α -smooth muscle cell actin (F). Note β -gal positive cells in the peri-infarcted myocardium (A, B, and E) were more elongated and mature compared to the cells in the midst of scar (C and D). In panel E, between β -gal positive cells (*thick arrow*) and β -gal negative cells (host cardiomyocyte, *thin arrow*), there was a gap junction positively stained for connexin 43 (*arrowhead*). (A–D, F) Arrows indicate β -gal-positive cells. Original magnification: $\times 400$ (A–F).

Figure 5. Cardiomyogenic differentiation of the MSCs. Picosirius Red staining to show connective tissue in red and muscle in yellow. β -gal positive cell (arrow) in the periscar showed blue nucleus in the yellowish cytoplasm. Arrowhead indicates epicardium of the heart. Original magnifications: $\times 100$ (A), $\times 400$ (B).

Figure 6. Echocardiographic data. Echocardiogram was performed before coronary infusion as baseline (0 weeks), 4 and 8 weeks after coronary infusion. EF, Ejection fraction of left ventricle (A); FS, fractional shortening (B); LVEDd, end-diastolic diameter of the left ventricle (C); LVEDs, end-systolic diameter of the left ventricle (D); dotted line, control group (n = 12); solid line, MSC group (n = 11). * $P < .05$ by unpaired t test.

Several in vitro and in vivo studies have confirmed the plasticity of MSCs to differentiate into cardiomyocytes.⁵⁻⁹ It has been suggested that to induce MSCs to differentiate into

cardiomyocytes, the cells need to be in close contact with the myocardial microenvironment, but within a scar they require in vitro treatment with 5-azacytidine prior to im-

plantation.^{6,7} Although the exact mechanism of action of 5-azacytidine has not been elucidated, Konieczny and colleagues²² proposed that embryonic cells contain a myogenic determination locus in a methylated state while in a transcriptionally inactive phase, which becomes demethylated and transcriptionally active with 5-azacytidine, causing the cells to differentiate into myogenic cells. Tomita and colleagues have reported that when implanted directly into a scar, only MSCs treated with 5-azacytidine showed positive staining for troponin I and myosin heavy chain, and they contributed to improved cardiac function by preventing scar thinning and chamber dilatation.⁶ The results of our more recent study comparing 5-azacytidine treated with non-treated MSCs appear consistent with their findings.²³ In the current study, we again confirmed the importance of the microenvironment for cardiomyogenic differentiation. The morphological appearance of the MSCs in the center of the scar was different to those in the periscar area, where the cells were surrounded by viable host cardiomyocytes. The latter showed well-organized contractile proteins, with small nuclei to cytoplasm ratio, and connected to adjacent host cardiomyocytes with connexin 43 positive gap junction, although the cells within the scar seemed less differentiated. We speculate that the cells found in the periscar area might have received more adequate in situ signals for cardiomyogenic differentiation than those within the scar. Besides the cardiomyogenic phenotype, we also found some labeled MSCs showing vascular endothelial or smooth muscle cell phenotypes integrated into vascular walls. They were likely involved in the angiogenesis and vasculogenesis in the healing process of myocardial infarction.¹⁰

It has been shown that MSCs implantation results in an improved cardiac function.^{6,9} Even though these studies showed improvement in cardiac function, exact mechanism remained unclear, as there was scanty evidence of gap junction formation between neomyofibers in the scar and the native myocardium outside of the infarct, making synchronous contraction unlikely. It has been speculated that function might be improved by the attenuation of adverse remodeling and enhanced contractility of cardiomyocytes. The improvement in regional function probably resulted from a combination of factors including myogenesis and angiogenesis.⁶ Clearly further mechanistic studies are warranted.

Limitations in the scope of this study also prevented us from addressing a number of important and relevant issues. Recently there was a major concern about the existence of true “pluripotent adult stem cells,” as Terada and coworkers²⁴ and Ying and colleagues²⁵ reported that MSCs could simply fuse with existing differentiated cells, with the appearance that they had differentiated into cells of other phenotypes. However, this notion had been challenged subsequently by a number of authors, such as that from Ver-

faillie’s group,²⁶ who conclusively demonstrated that a single adult stem cell derived from the bone marrow could be guided to differentiate into various specific phenotype, in the absence of any preexisting differentiated cell to which an adult stem cell could fuse with. Tomita and colleagues²⁷ cocultured MSCs with cardiomyocytes in vitro and reported that the MSCs expressed various phenotype specific proteins, such as troponin I-C, connexin-43, and atrial natriuretic factor, in sequence rather than simultaneously. This observation also supports that MSCs undergo differentiation rather than fusion. This is because in a fusion, the cardiomyocyte that fuses with MSC should already contain all the phenotype-specific proteins, so that it is not possible for these markers to become detectable one by one over several days. We have discussed the phenomenon of such milieu-dependent differentiation in greater detail elsewhere.²⁸

Another important limitation in this study is that we did not attempt to use specific cell markers to characterize and define the preimplant properties of MSCs, as we stated earlier. It should be noted, however, that the clonal definition of MSCs is not fully standardized yet at present, as the investigators still use various different cell markers, cellular culture, and isolation techniques in their studies. Likewise, the specific molecular signals for MSC homing and differentiation remain unknown and will also need to be elucidated in future studies.

Although intracoronary injection of cells provides certain advantages, coronary embolism leading to myocardial infarction is a major concern with this delivery approach.²¹ In our preliminary study, we bolus injected a greater quantity of cells (eg, 5×10^6) and found higher mortality rate (more than 50%). As soon as the MSCs were infused, hearts became pale and stopped beating due to embolism. Therefore, to prevent embolic myocardial damage, measures to avoid cellular aggregation, as well as slower or multiple infusions of diluted cells, need to be considered. Clinically, this approach may be most suited for patients who have heart failure caused by diffuse cardiomyopathy.

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Discussion

Dr T. Rosengart (Evanston, Ill). I congratulate the authors on a beautiful presentation. They certainly are pioneers in the field, together with Dick Weisel and Philippe Menasche, and really have led a growing body of evidence suggesting some very exciting work in the possibility of cell transplant.

I have four questions for Dr. Saito. The first question involves the use of 5-azacytidine to drive differentiation. Your histology very elegantly shows apparent differentiation of cell lines based upon the localization either to scar or periscar, appearing to go to a myocyte differentiation or a fibroblast differentiation. Based upon these findings, do you think that additional 5-azacytidine would be efficacious or a more prolonged predifferentiation prior to transplant?

In contrast, there certainly are a number of studies now suggesting that undifferentiated cells, bone marrow or otherwise, may equally be efficacious in driving myocardial remodeling. Given concerns potentially by the US Food and Drug Administration or other regulatory agencies in going to clinical trials in terms of mutations, potential mutations, and complications using 5-azacytidine, how do you see the role of using this or other differentiating factors?

Dr Saito. We have not seen it.

Dr Rosengart. Do you think that the azacytidine plays an important role, or do you think that simply transplanting undifferentiated cells will be as efficacious?

Dr Saito. Yes, I think so. We have never seen myotube formation or pulsating cells in a culture dish even after treatment with 5-azacytidine. Possibly I don't think 5-azacytidine worked. Therefore, we reconfirmed the importance of a microenvironment.

Dr Rosengart. The second question involves the vascular delivery, the arterial delivery, and certainly this has advantages in terms of global dissemination, spreading of the cells throughout the myocardium. The concern, of course, as you have shown in your histology, is the risk of microembolization. Have you looked at creatine kinase levels or other evidence of myocardial injury? Is that a potential disadvantage of this delivery technique?

Dr Saito. Yes, it is a very important point. Actually we have never checked the creatine kinase level, but if we infused more number of cells, the mortality was much higher. For example, if I infused 5 million cells as a preliminary study, more than half of the animals died immediately after cell infusion. That was I think due to microembolization. I have never seen a creatine kinase level.

Dr Rosengart. The third question involves your echo data, and it appears very clear that when you look at your ejection fraction and your end-diastolic and systolic volumes that you do have what appears to be a stabilization of geometry or compliance. When you look at your fractional shortening data at the 8-week time point, you appear to have an improvement in function that is not consistent with your other parameters. Do you have any explanation why these parameters appear to be different?

Dr Saito. Ejection fraction was calculated using LVEDd and LVEDs. That was presented. But when calculated, I also don't understand why only at 8 weeks marrow stromal cells show, how to say, very improved cardiac function. But basically this value,