The effect of cell therapy using a human mesenchymal stem cell injection on left ventricular systolic function in rat myocardial infarction model

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A Dissertation

Submitted to the Department of Internal Medicine

and the Graduate School of Yonsei University

in partial fulfillment of the

requirements for the degree of

Ph. D. in medical Science

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July 2005 of submission

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2005 년 7 월

저자 씀

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ABSTRACT

The effect of cell therapy using a human mesenchymal stem cell injection on left ventricular systolic function in rat myocardial infarction model

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(Directed by Professor Junghan Yoon)

Objective: Adult mesenchymal stem cells (MSC) have the potential candidate for cell replacement therapy to treat many myocardial diseases. It is thought that MSC do induce minimal immunoreactivity even to xenografts. We investigated the effect of cell therapy using a human MSC injection on left ventricular function and its possible mechanism in rat myocardial infarction model.

Methods: Bone marrow was aspirated from healthy human to get adult MSC. We isolated and grew MSC using previously proven method. These MSC were confirmed by MSC surface markers using flow cytometry. We performed 2

separated sets of animal procedure. First set (n=25) was performed for the assessment of LV function and neovascularization and second set (n=10) was performed for the evaluation of hMSC engraftment using GFP immunostaining.

An infarction was created by ligating the left anterior descending artery (LAD) of 25 Sprague-Dawley rats .In first experimental set, animals are divided into the 2 groups; control and MSC group. After LAD ligation, 1 x 10^6/kg MSC or saline were injected 3 times around the infarcted area using a 28-gauze syringe in volume of 0.3ml. At 28 days, a functional assessment of LV using transthoracic echocardiography (VIVID 7, GE medical, 12 MHz probe) was performed before sacrifice. After sacrifice of animals, hematoxylin-eosin staining and vWf staining were performed for histological analysis. In second set, animal was sacrificed 2 weeks after GFP incorporated MSC transplantation and immunohistochemical staining was performed in 10 animals.

Results: Periprocedual animal mortality was 28%. There were no significant difference in mortality between the two groups (control; 30.7%, MSC; 25% rats, $p<0.05$). After 28 days, there was a significant improvement in the fractional shortening of LV in the MSC group compared with that in the control group (22.1±3.9 vs. 39.2±4.5%, $p=0.023$). The number of VWf (von Willebrand factor) positive cells and the density of microvessels on the border

area was significantly higher in the MSC group (4.6±0.6 vs. 8.9±1.1 vessels/HPF, p=0.021). In 2nd set, immunfluorescent study revealed engrafted cells expressing a smooth muscle phenotype (alpha actinin) and GFP (green flurorescence protein) in MSC group.

Conclusion: This study demonstrates that transplantation of human MSC induces myocardial angiogenesis in a rat model of myocardial infarction as evidenced by increased blood vessel density. Furthermore, successful engraftment of human MSC transplantation leads to a significant improvement in the post-infarction left ventricular systolic function.

__Key words: Cell; Transplantation; Myocardial Infarction: Stem cell

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1. Introduction

Adult cardiac muscle, unlike skeletal muscle, lacks the ability to regenerate after ischemic injury, and the death of cardiomyocytes might be a cascade that results in heart failure.[1-2)] Cardiac cellular transplantation techniques have made significant progress, and a variety of cell types have been proposed as useful candidates for cell therapy.[3-5)] Bone marrow contains multipotent adult stem cells, which have a great capacity of directionally differentiating to a myocardial cell.[6)] Although many cell types from the bone marrow contribute to the organ repair in infarction model, bone marrow

mesenchymal stem cells (MSC) have the greatest potential to repair the myocardium. Transplantation after ex vivo culture expansion of MSC is mandatory to meet the dose requirements that have been effective in animal models, because few MSC can be obtained by bone marrow aspiration. The growth rate of MSC depends on the density of cells. Unless cultured for prolonged periods, MSC do not show senescence and apoptosis. Thus, MSC can be tremendously expanded in number within a reasonable period.[7-8] MSC have been also used in a model of cardiac cellular transplantation, showing that these cells could not only differentiate into the myogenic cells but also enhance angiogenesis.[9-10] Also, many articles were reported about weak MHC expression for MSC.[12-14] Some articles[14-15] were reported that MSC can initiate the activation of alloreactive T cells, but do not elicit T cell proliferative responses due to active suppressive mechanisms. A report demonstrated the effect for human cell transplantation without immune suppressive treatment in an ischemic brain rat model suggesting that adult human MSC might do not induce immunoreactivity even to xenografts.[16]

MSC have the potential candidate for cell replacement therapy to treat myocardial diseases. There was no paper which has been applied with human MSC to evaluate for the effect and mechanism of cell therapy in animal

infarction model. Therefore, we investigated the effect of cell therapy on the repair of infarcted myocardium using a human MSC injection on left ventricular function and its possible mechanism in rat myocardial infarction model.

2. Methods and Materials

We performed 2 separated sets of animal procedure. First set was to evaluate LV function and neovascularization. And second set was performed to define graft sucesst of the hMSC in the infart zone using GFP immunostaining.

2.1. Isolation of hMSC

A bone marrow aspirate was collected from the posterior iliac crest of healthy volunteers with a syringe containing 6000 U heparin. The marrow sample was washed with Dulbecco's PBS, and cells were recovered after centrifugation at 900*g*. Bone marrow mononuclear cells were isolated by Ficoll density centrifugation. Mononuclear cells (1x 10^6/ml) were placed in a 175cm^2 flask (Falcon, Franklin Lakes, NJ) and were cultivated in low-glucose Dulbecco modified eagles' medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Irvine, CA) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) in a humidified incubator at 37°C under 5% CO_2. After 5 days, nonadherent cells were removed by replacing the medium. Attached cells developed into colonies within 5 to 7 days. When these primary cultures of MSC reached 80% confluence, the cells were harvested using 0.25% trypsin and subcultured (Fig. 1). MSC were

culture expanded to reach 1x 10^{7-8}/ml cells within a relatively short period of culture (mean: 30.8±5.5 days; range, 23–37 days). Based on body weight, 1 x 10^6 cells/kg/rat is the rat dose equivalent to the dose that was effective in a rat model of other disease (1 x 10^5 ~ 3 x 10^6cells/rat).

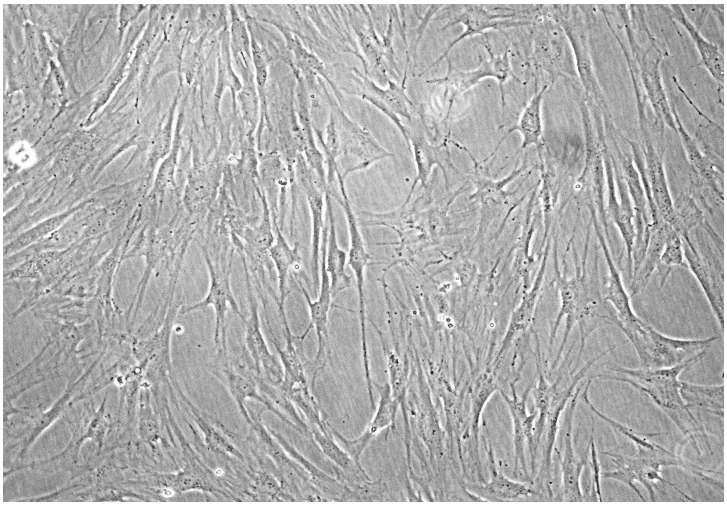

Figure 1. Human mesenchymal stem cells in culture appear fibroblastic and homogenous in size and morphology after 2nd passage (x100 magnification).

2.2. Cell Preparation for cell transplantation and MSC labelling

Because stem cells are highly likely to be differentiated, the surface expression of SH-2 (Src homology, CD105) and SH-4 on culture-expanded MSC was measured using flow cytometry (FACScan; Becton-Dickinson, Rutherford, NJ) before infusion. Every harvest of MSC showed a homogenous

population of cells with high side and forward scatter and high expression levels of SH antigens (>91% of cells; Fig. 2). These cells did not express CD34, CD45, human leukocyte antigen-D related, or class I human leukocyte antigen (data not shown). Cell viability was determined by trypan blue staining at the end of the harvest and before infusion; viability was greater than 95%. Mycoplasmal contamination in any of the flasks was tested. On the day of injection, the cells were harvested using trypsin, washed with phosphate-buffered saline, and resuspended.

To evaluate the cell tracking and successful engraftment of hMSC**,** MSC were transplanted into the infarct zone after the induction of myocardial infarction. MSC transfected with the green fluorescent protein (GFP) were cloned before transplantation to facilitate subsequent identification, briefly described as follows. MSC transfection was achieved by the ViraPower^{tm} (Invitrogen corp. CA, USA) lentiviral expression system (vector; pRRLsin.PPT-hCMV). Briefly, the GFP vector were added to MSC and cultured for 4 days prior to 70% of GFP positive cells were detected by flow-cytometry. After quickly washing the cells to remove unincorporated GFP was selected with antibiotics, we detached MSC from the bottom of the flasks with 0.25% trypsin and 1 mM EDTA and then resuspended MSC in serum-free

medium for transplantation. Cell dosage of GFP incorporated MSC and method of injection was performed with same procedure as the method of first experimental set. Two weeks after MSC transplantation, assessment of myocardial expression of GFP by fluorescence-microscopy in injected area was performed.

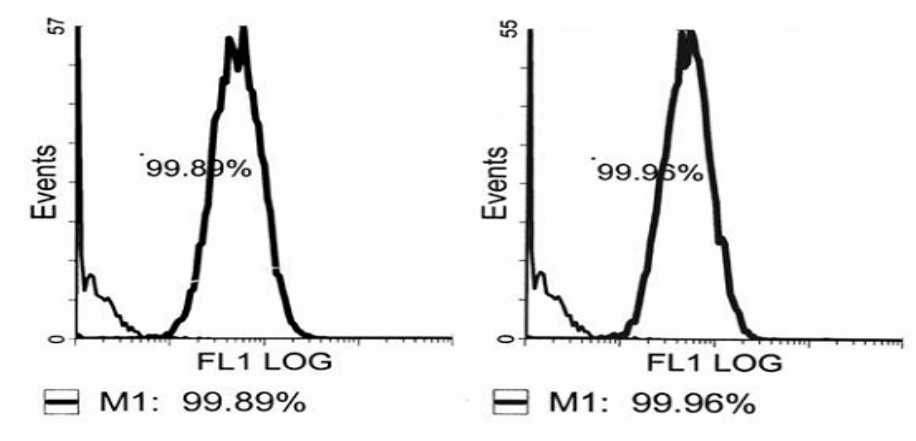

Figure 2. Flow cytometric analysis of the mesenchymal stem cells of each patients with antibody directed against SH-2 (CD105).

2.3. Induction of myocardial infarction

Under anesthesia with ketamine (50 mg/kg i.p.), male Sprague–Dawley rats weighing 250 ~300 g were intubated with an 18-gauge intravenous catheter with a tapered tip via tracheotomy. Rats were mechanically ventilated on a rodent ventilator with room air. Through a left thoracotomy in the fourth

intercostal space, the myocardial infarction was induced by ligating the left anterior descending coronary artery (LAD) 2–3 mm from the tip of the left auricle with a 6–0 polypropylene (Ethicon, NJ). Successful performance of coronary occlusion is verified by observation of the development of a pale color in the myocardium distal to the ligation.

2.4. Cell transplantation

After successful ligation of LAD, MSC (1 x 10^6/kg) or saline were injected 3 times around the infarcted area using a 28-gauze syringe in volume of 0.3ml depending on the study group.

2.5. Assessment of LV function with echocardiography

In first experimental set, LV function was studied by 2D echocardiography in animals four weeks after cell transplantation. The rats were placed in the supine position after ketamine anesthesia. Transthoracic B and M mode measurement with a commercially available 12 MHz transducer system (VIVID 7, GE medical) was performed using a standard technique including parasternal long-axis views and parasternal shot axis view at the level of the mitral valve, the aortic valve and the papillary muscle. Left

ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were calculated by means of the single-plane area-length method. Left ventricular fractional shortening (%LVFS) were then calculated (LVFS= (LVEDD−LVESD)/LVEDD). Anterior and posterior end-diastolic and end-systolic wall thickness and LV internal dimensions were measured according the American Society for Echocardiography leading-edge method. LV end-diastolic (LVEDV), end-systolic volumes (LVESV), and ejection fraction (LVEF) were calculated by using the modified Simpson's single plane rule from long-axis view. End-diastole was selected as the largest LV area and end-systole as the smallest.[17] All measurements were averaged on three consecutive cardiac cycles and were analyzed by two independent observers who were blinded to the treatment status of the animals.

2.6. Sacrifice and histologic staining

H & E, Masson trichrome, and von Willebrand factor staining were performed in first experimental set and immunofluorescent staining was performed using the cardiac myocyte antibody in second experimental set. In first set, each animal was anesthetized and prefused through abdominal aorta with 100 ml saline and 100 ml 4% paraformaldehyde in 0.1 mol/L phosphate-

buffered saline. After 24 hours of fixation in 4% paraformaldehyde, the heart was cryopretected with 30% sucrose for 24 hours and cut by cryostat (Leica CM 1900) into 30-um sections. The tissue sections were fixed for 2 minutes at room temperature with either acetone or acetone/methanol 50%/50% vol/vol and then incubated with PBS containing 5% goat serum. After thorough washing, the primary antibody was applied for an additional 60 minutes. rabbit polyclonal anti-Factor VIII related antigen/von Willebrand factor (vWf) antibody (1 : 80, NeoMarkers, USA) mouse monoclonal antibody. In case of vWf, biotinylated anti-rabbit IgG (1 : 200, VECTOR LABRATORIES, USA) was applied as the second antibody, followed by DAB identification.

Imaging was performed with microscope. Images from the entire sections were acquired using digital camera system (Diagnostic Instrument, MI). The slides were processed to morphometry using a computerized image analysis for quantitative assessment of vascular density in myocardium. In the images, red cells in blood vessels were stained red while collagens in infarct zone stained blue. The number of blood vessels was counted in 10 random fields (magnification 20×). The average of the 10 high power fields (hpf) was calculated and the vascular density was defined as blood vessels/hpf.

In second experiment, we performed same perfusion and tissue preparation techniques as first experiment. These included either mouse monoclonal anti-α-actinin (sarcomeric) antibody (1 : 400, Sigma, USA) or mouse monoclonal anti-troponin T (cardiac) (cTnT) antibody (1 : 200, Neo-Markers, USA). Rhodamine red dye-conjugated goat anti-mouse IgG (1 : 50, Jackson Immunoresearch, USA) was applied as a second antibody and fluorescence imaging was performed with a Zeiss Axiovert equipped for epifluorescence or a Nikon PCM 200 confocal microscope.

2.7. Statistical analysis

All values are expressed as mean±S.E.M. Statistical comparison of the data regarding capillary density, LV function and other numeric data was performed using a Student's *t*-test between control group and MSC group. $P<0.05$ was considered significant.

3. Results

3.1. Experimental sets

Periprocedual animal mortality was 28% after 4 weeks of cell transplantation in first experimental set (7 / 25 rats). The number of animals used for analysis was 9 in control group and MSC group, respectively. There were no significant difference in mortality between the two groups (control; 4/13, MSC; 3/12 rats). In second procedure, periprocedual animal mortality was 30% after 2 weeks of cell transplantation (3 / 10 rats) (Fig 3).

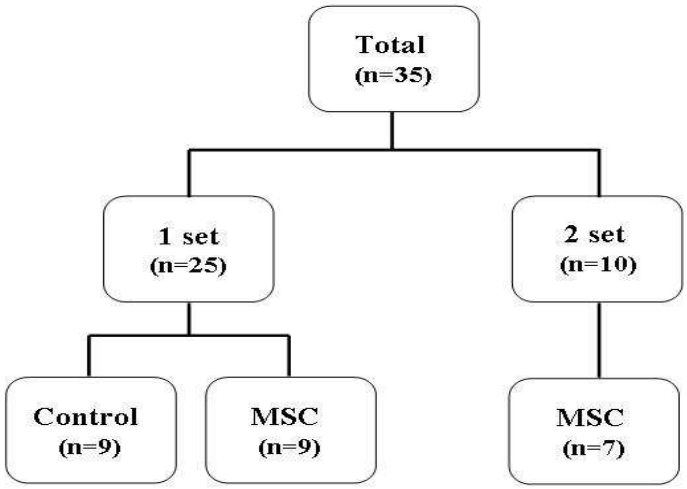

Figure 3. Diagram of animal procedures.

3.2. Effect of MSC transplantation on LV function

There was no significant difference in the level of LVESD (6.8±2.1 vs. 7.2±1.9 mm) and LVEDD (9.1±2.4 vs. 9.7±2.9 mm) between the two groups (p=NS). However, a significant improvement of fractional shorting was observed in the MSC group compared with control group (22.1±3.9% vs. 39.2±4.5% at 4 weeks post-transplantation; p=0.023, Fig. 4). Calculated LV ejection fraction was significant improvement in MSC group (48.1±5.7%) compared with control animals (35.2±3.1% $P < .05$, Table 1).

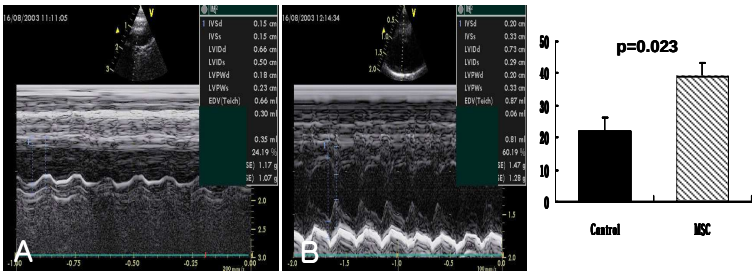

Figure 4. Echocardiographic data of control group and MSC group. M-mode echocardiograms showed that left ventricular (LV) anterior wall motion was preserved in the MSC (B), but not in the control hearts (A).: LV fractional shortening(FS) was significantly higher in the MSC compared to the control group (right graph;p<0.05).

Table 1. Echocardiographic Data

	Control group	MSC group	p value
LV end-diastolic diameter (mm)	9.7±2.9	9.1±2.4	0.73
LV end-systolic diameter (mm)	7.2±1.9	6.8±2.1	0.87
LV diastolic ID (mm)	7.8±0.2	7.3±0.3	0.10
LV systolic ID (mm)	3.8±0.3	4.6±0.4	0.053
LV fractional shortening (%)	22.1±3.9	39.2±4.5	0.023
LV ejection fraction (%)	35.2±3.1	48.1±5.7	0.012
Septum thickness (mm)	1.91±0.01	1.88±0.03	0.051
LV end-diastolic volume (mL)	0.73±0.03	0.63±0.04	0.34

LV: left Ventricular, ID: internal diameter

3.3 Effects of MSC transplantation on neovascularization

Neovascularization, as defined by vascular densities, was quantified in the grafted MSC group and control group. Interestingly, angiogenesis was significantly increased in the border zone of the scar compared to the control group (4.6± 0.6 vs. 8.9±1.1, capillaries per high-power field (hpf), p=0.021, Fig. 5, 6). And there was no increase in capillary density in scar zone in the MSC group compared to control group (2.7±1.5/hpf vs. 2.7±1.0/hpf, *n*=7). Furthermore, the integration of vWf-postivie cell in the new blood vessels was confirmed as a component of vessel formation (Fig. 5C).

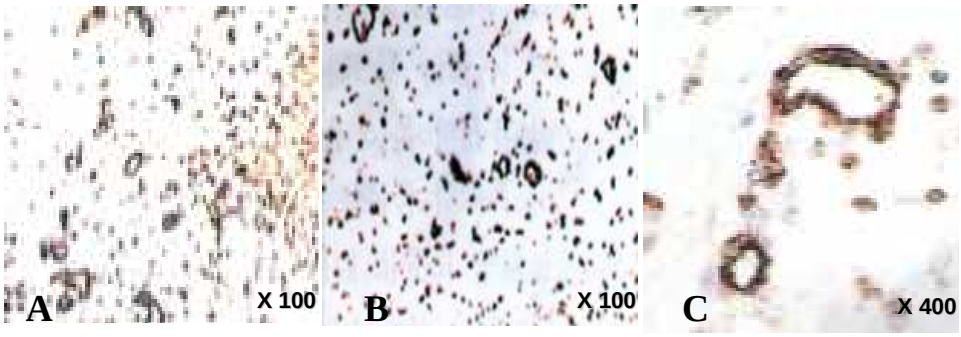

Figure 5. Photomicrographs of adult rat hearts transplanted with human MSC at 4 weeks after left coronary artery occlusion. Vascular density was significantly greater in MSC group (A) than in control levels (B) (*P*=0.021). VWf positive vessel cells in MSC group were appear in higher (C, ×400 magnification).

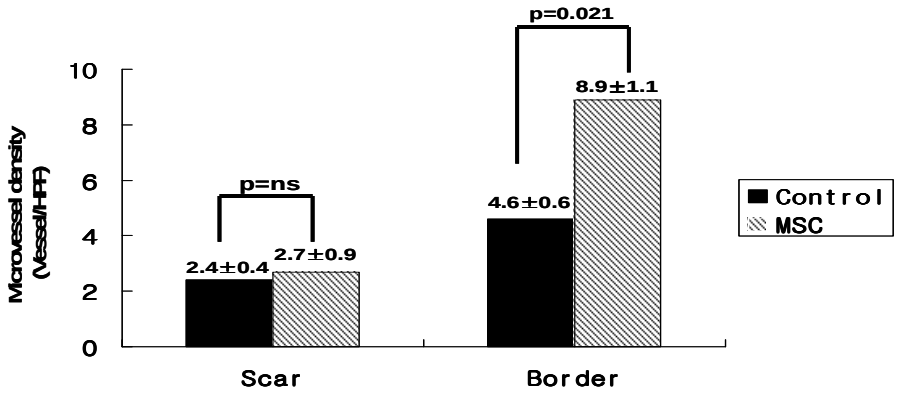

Figure 6. Vessel density was increased mainly in the border of infarct scar where the implanted cell clusters were observed.

3.4. GFP expression and immunostaining in myocardium after cell transplantation

As shown in Fig 7, myocardial expression of GFP in the MSC transplantation group was appeared 2 weeks after treatment. The GFP-positive cells and alpha actinin positive cells were mainly localized in border areas.

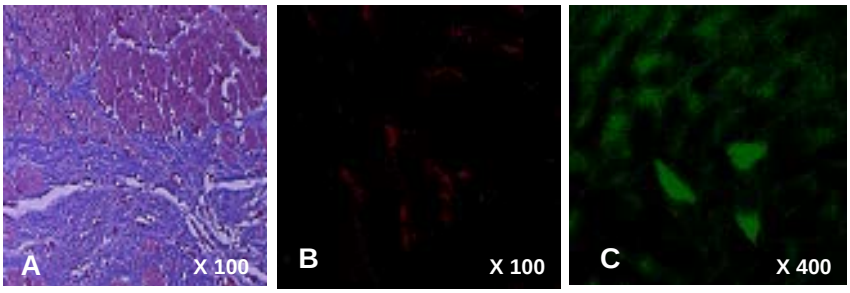

Figure 7. MT staining showed a cluster of implanted cells at the anterior border area of MI scar (blue stain denotes the fibrous tissues in MI scar) (A; x100) magnification). Immunohistochemical staining revealed sarcomeric α-actinin expressions in implanted MSC (B; x100 magnification). A furomicroscpic finding of GFP in myocardium demonstrates enhanced expression of GFP in ischemic myocardium (C; x400 magnification).

4. Discussion

This study demonstrates that transplantation of human MSC transplantation leads to a significant improvement in the post-infarction left ventricular systolic function. Although the exact mechanisms underlying LV function improvement by MSC are unclear, we think that angiogenesis and successful engraftment of MSC play a major role on improvement of LV function.

Recently, it became clear that a small population of MSC in the bone marrow includes putative adult stem cells with important functional features, giving rise to a wide variety of connective tissues, including bone, cartilage, muscle, fat, hemopoiesis-supporting stroma, and perhaps neuronal cells. Adult mononuclear bone marrow cells contain few (<1%) stem cells. [18-19] Moreover, therapeutic injection distributes MSC to organs, which further decreases the number of cells that reach the myocardium.[20] And, some experiments have indicated that as low as 3% of MSC administered by direct injection persist after 2 weeks.[21] Administration of higher numbers of cells results in only modest augmentation of long-term engraftment. The limited number of available MSC requires that there be a process to isolate and increase the

number of these cells ex vivo. Although rare (1 per 10^6 bone marrow mononuclear cells) in bone marrow, MSC proliferate rapidly in vitro (48–72 hour in doubling time) and have been expanded by more than 60 cell doublings.[22, 23] Considering these facts, we decided to transplant MSC at a dose that had been shown to be effective in rats. Although the stem cells are highly prone to differentiation, our flow cytometry data indicated that the culture-expanded MSC had a high level of expression of MSC surface markers (SH-2 and -4).

Intuitively, one might expect allogeneic MSC (allo-MSC) would stimulate T cell proliferation and that donor MSC would be recognized by responder T cells and rejected by a recipient host. However, experimental evidence indicates this may not be the case. MSC have been shown to inhibit T cell proliferation in several laboratories.[24,25] Overall, MSC appear to prevent maturation of T cells to their cognate antigen or other antigens by a direct cell–cell effect and a soluble factor or factors. Several studies have used allo-MSC in vivo, and experience suggests the allo-MSC is not rejected and may have positive effects on engraftment of third-party cells or tissue.[26] An alternative approach is use of allogeneic stem cells. Collectively, current studies on the interactions between MSC and T cells support the potential use of allo-MSC in

cell therapy. Along with studies of allo-MSC introduced into infarcted heart models and encouraging early clinical results, the future use of allo-MSC has significant clinical potential.[27] In this study, a reliable xenograft MSC transplantation model was established in rats. Thus, although main focus of our study did not verify the possibility for xenograft, it is a clinically relevant model for xenograft cell therapy, which is substantially different from previously reported methods using syngenic rats to simulate autologous cell transplantation.[28] Also, this study demonstrates that human MSC was successful engraftment and human MSC engraftment might to contribute to the improvement on the cardiac function in such a setting, if it is impossible to do in clinical setting, but, our study had limitation to assess immune response or rejections. Several laboratory works need to characterize the interactions between MSC and cells of the immune system.

We provide evidence for MSC ability to improve cardiac function after transplantation, as evaluated by non-invasive echocardiography for up to 4 weeks. We thus propose that the enhancement of cardiac function in MSC therapy may be attributed to the following mechanisms: first, MSC possess the capacity of self-renewal that can maintain the long-lasting effect of angiogenesis. Angiogenesis can improve myocardium perfusion and preserve

stunned cardiomyocytes.[29,30] Second, the enhancement of LVEF is due in part to MSC being integrated into healthy host tissue to form new myocardium, though the ratio of differentiation to cardiomyocyte is limited. It might also be beneficial in reducing scar expansion.[31] In our study, we found the neovascularization decreased at the border areas. But, there were no improvement in micro-vessel formation at scar area. The reasons for this result were considered mainly permanent myocyte necrosis with decreased blood vessel formation because of permanent artery occlusion of our LAD ligation model. Probably, this situation was different from previous temporary occlusion model. This may be explained by the hypothesis that the neovascularization induced by MSC involves paracrine factors secreted by MSC to facilitate vessel sprouting, growth and incorporation of MSC into newly forming blood vessels by differentiation of MSC in situ.[32] But, we did not check simultaneously between the tracking of engraftment (after 2 weeks) and LV function (after 4 weeks cell transplantation). Also, alpha-actinin positive cells presented myocyte-specific antigen, but its characteristics and function was not evaluated in this study. Further evaluation for cell characteristics with physiologic assessments will be needed. One of the problems inherent to the study of cellular cardiomyoplasty is the difficulty in

tracking the movement of implanted cells. MSC localization in vivo with either MRI or gamma imaging is of tremendous value to those attempting to demonstrate the biodistribution or persistence of MSC cardiomyoplasty.[33, 34] Our results indicate that the transplanted cells reach the lesion zone as indicated by GFP fluorescence but we assessed the accurate cell engraftment quantification. However, the inability of the techniques to accurately quantify the number of MSC is troubling to those seeking to optimize the therapeutic use of MSC. Also, we did not check simultaneously between the tracking of engraftment and myocyte specific immuno-staining (double staining). Therefore we did not confirm the exact mechanism for LV functional improvement, which is successful engraftment or other effects. These points would be a significant limitation in this study. Further evaluation for exact mechanism and cell tracking with quantification will be needed.

In conclusion, the transplantation of human MSC improves left ventricular systolic function, increased microvessel formation and expressed cardiac muscle proteins in rat myocardial infarction model. MSC engraftment and angiogenesis might to contribute to the improvement on the cardiac function in this a setting.

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국문요약

흰쥐의 급성 심근 모델에서 좌심실 수축 기능에 대한 사람의 중간엽 줄기 세포를 이용한 세포 치료의 효과

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배경 및 목적 :

성인 중간엽 줄기세포(mesenchymal stem cell: 이하 MSC)를 이용한 세포치료가 새로운 방법으로서 최근 각광을 받고 있다. 하지만 기존의 실험 연구는 동물 세포에서 동종간의 심 기능 개선 효과를 증명하였고 실제 사람의 MSC 를 획득하여 동물 심근 경색 모델에 적용한 예는 드물다. 이에 본 연구를 통하여 실험실내에서 치료 목적으로 사람의 MSC 를 배양 및 세포 수를 증폭시켜 흰쥐의 심근 경색 모델에 적용하여 그 치료 효과를 입증하고 그 기전을 알아보고자 하였다.

방 법 :

건강한 성인 골수에서 세포를 획득하여 기존의 확립된 방법을 이용하여 MSC를 실험실내에서 배양하여 증폭시켰고 이를 flow-cytometry로 확인하였다. 또한 흰쥐 (n=25, BW=294g)의 관동맥을

결찰하여 심근 경색을 유발하여 무작위로 MSC (1 x 10^6/kg)를 이식한 군과 대조 군으로 나누어 심근경색의 중앙 부위와 주변 부위에 총 3회에 걸쳐 주입(총 0.3ml)하였다. 4주후 희생하여 심초음파도를 이용한 기능적 검사 및 조직 검사를 시행하였고 일부 흰쥐 (n=10)를 이용하여 GFP (green fluorescent protein)를 전처치한 2주 후 조직 검사 및 면역 화학 검사를 시행 하였다.

결 과 :

심근 경색 후 실험 동물의 사망률은 28%였고 세포 이식 4주 후 심초음파를 이용해 측정한 좌심실 내경은 MSC군과 대조군 사이에 유의한 차이가 없었으나, 좌심실 분획 단축률은 MSC군에서 유의하게 증가하였다 (22.1±3.9 vs. 39.2±4.5%, p=0.023). 이식 세포의 군집 주위에서는 von Willebrand factor에 염색되는 많은 혈관들을 발견할 수 있었으며, 경색 주변부의 미세혈관 개수는 MSC군에서 대조군과 비교하여 유의하게 높았다 (4.6±0.6 vs. 8.9±1.1 vessels/HPF, p=0.021). 또한, GFP로 전처치된 MSC는 경색 주변부에 발현 되었고, sarcomeric α-actinin이 발현되는 많은 수의 이식 세포들을 확인할 수 있었다.

결 론 :

흰쥐의 심근경색 모델에서 이식된 사람의 MSC는 심기능을

향상시켰고 경색 주변부 미세혈관의 형성을 증가시켰으며, 수축 조절 단백질을 발현하였다. 본 연구의 결과는 MSC의 세포내 engraftment가 심근 기능의 회복에 기여 했을 것이라 생각된다.

핵심 되는 말; 세포 ; 이식 ; 심근경색 ; 줄기 세포