Improvement of adherence and therapeutic effect of PSGL-1 transfected mesenchymal stem cells in myocardial infarction model

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

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Mesenchymal Stem Cell(MSC) is used as a therapeutic agent in many clinical fields. These stem cell therapies are also used in heart diseases

such as myocardial infarction and heart failure. A common problem in many of the existing stem cell therapies is the fact that the injected stem cells are not properly transferred to the diseased area and are dispersed to other organs. In fact, it has been reported that the efficiency with which stem cells injected into the blood vessels migrate to the lesion site is less than 2%, and the remaining cells are found mainly in the lungs or kidneys and also disappear within a short period(4 weeks). In order to increase these low engraftment rate of MSCs, we have applied to the stem cells a protein involved in the mechanism of migration of the immune cells circulating in the blood vessels during the initial immune reaction to the inflammatory site. When tissue damage occurs such as cell necrosis, a signal substance like interleukin is secreted from the relevant site to stimulate vascular endothelial cells, and proteins such as selectin and CAM are expressed on the surface. P-Selectin Glycoprotein Ligand-1(PSGL-1) or L-selectin, which is expressed on the surface of immune cells, binds to proteins expressed by stimulation in vascular endothelial cells and it is possible to increase the number of stem cells migrating to the lesion site by moving on the inner wall of the vessel and showing a decrease in the moving speed. We use this principle, to increase MSC therapeutic efficiency, make PSGL-1 express on MSC surface by gene transfection.

For gene transfection, viral or non-viral carrier is used to deliver the gene. Our final goal is clinical application, so PSGL-1 transfer was performed using non-viral carrier ABP. In order to improve low transfection efficiency, which is a problem of non-viral carrier, we separated the transfected and untransfected cells by flow cytometry

through GFP(Green Fluorescent Protein), a widely used fluorescent protein. Through this method, the effect of the gene was increased by using only the transfected cells for treatment. After that, MSC expressing PSGL-1 was injected into myocardial infarction rat model and verified the effect of transgenic stem cells in each period and step. After injection of stem cells into the myocardial infarction model, we harvested heart tissue 3 weeks later after operation, performed Immunofluorescence staining to measuring remained MSC on infarct site. And also confirmed the infarct rate and petrification rate were reduced thorough trichrome staining, TTC staining, and H & E staining. In addition, confirmed the improved cardiac function after stem cell therapy using echocardiography.

Key words: P-Selectin Glycoprotein Ligand-1, stem cell therapy, cell transfection, cell implantation, myocardial infarction

I INTRODUCTION

Cardiovascular disease is one of th major causes of death around the whole world[1][2]. Serum deprivation and hypoxia cause by restricted blood flow in myocardial infarction(MI) lead to cardiomyocyte death[3].

Cell therapy using mesenchymal stem cells(MSCs) have attracted much attention for tissue regeneration, especially for myocardial infarction(MI)[4]. So many clincal trials are performed using mesenchymal stem cell[5]. It has been presented that the paracrine factors secreted by MSCs, rather than MSCs commitment to cardiac lineage, contributes to heart repair after implantation of MSCs[6]. However, therapeutic benefit of MSC implantation has limitation due to its poor engraftment and survival of after injection[7]. In fact, fluorescence marked MSC injection at heart demonstrated very low surviving efficiency after few days[8].

To enhance surviving efficiency of MSC, I used adherence molecules. First, conformed P-Selectin expression in ischemic heart[9]. And found P-Selectin glycoprotein ligand-1(PSGL-1), can bind to P-Selectin. PSGL-1 is protein that express immune cell surface, and induce some protein like selectins and CAMs in damaged tissue[10]. So immune cells move to damaged tissue and repair this region.

Therefore, expressing PSGL-1 to MSC surface can repair myocardial damage. In fact, when implantation MSC, high MSC surviving improved heart function and therapeutic effect in myocardial infarction by paracrine effect of MSCs[11]. Performed transfection MSC with PSGL-1, and confirmed expressing PSGL-1 at MSC surface[12]. And confirmed binding

PSGL-1 and P-selectin in vitro by shear stress[12]. Next, I thought that implatation of PSGL-1 transfected MSCs would increase MSC surviving and significantly improve therapeutic efficiency in myocardial infarction by MSC's paracrine effect(Figure 1).

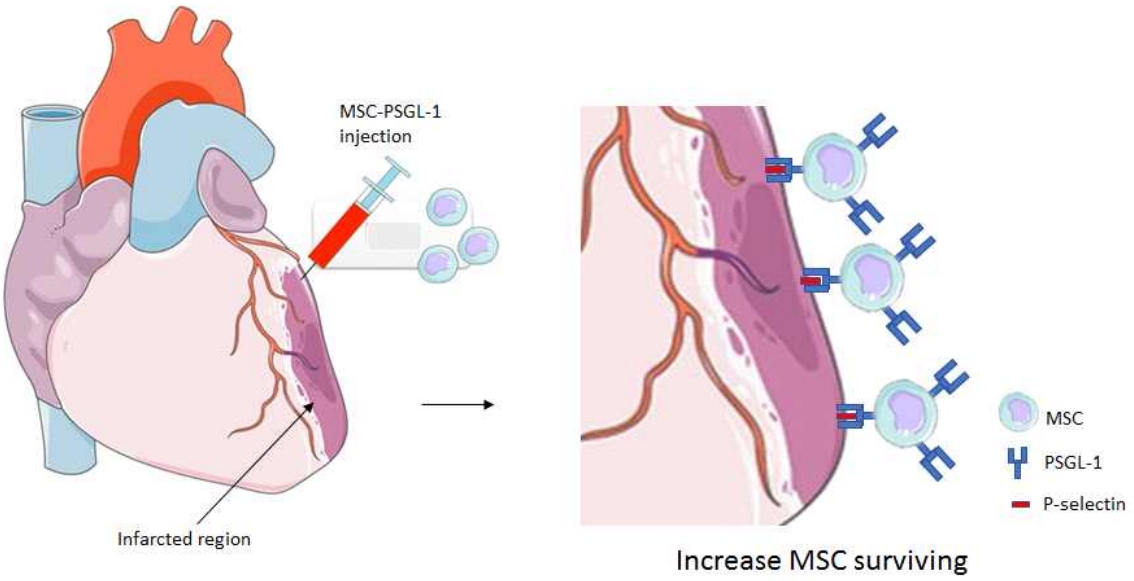


Figure 1. MSCs transfected with pEF1/Hisc C ::PSGL-1::EGFP increase MSC surviving efficiency after MSC injection into the infarcted myocardium. Transfeted MSC with PSGL-1 inject infarcted myocardium boarder zone. PSGL-1 on MSC surface bind P-selectin on infarcted region and enhance MSC surviving.

Ⅱ MATERIALS AND METHODS

2.1 Animals

Spraque-Dawley(SD) rats from ORIENT BIO(Seongnam, Korea) were cared for according to the Association for Assessment and Accreditation of Laboratory Animal Care International system. All animal experiments confirmed to the International Guide for the Care and Use of Laboratory Animals. All experimental procedures were examined and approved by the Animal Research Committee of Yonsei University College of Medicine.

2.2 MSCs isolation and culture

MSCs were isolated from four-week-old male Sprague Dawley rats (100±5g) by flushing the femurs and tibias with low glucose-Dulbecco's modified Eagle's medium(DMEM)(Welgene) supplemented with 10% fetal bovine serum(FBS)(Gibco) and 1% penicillin/streptomycin solution (Gibco), using 19-gauge needle. Flushing media were centrifuged at 1,600rpm for 5 minutes and suspended in complete medium. And then, suspension cells media were loaded onto a Ficoll-Paque(GE Healthcare Life Sciences). After being centrifuged at 1,600rpm for 30min, the following three layers(top, middle, and bottom) were visible. Transferred middle layer(mononuclear bone marrow cells) in new 15ml conical tube and washed twice with PBS. This mononuclear bone marrow cells were seeded onto 100mm culture dishes. After 72hrs incubation , non-adherent cells were discarded, and the adherent cells were thoroughly washed twice with PBS and changed fresh

MSCs media. This wash step was repeated every 2 days for 1 week.

2.3 Preparation of rat myocardial infarction(MI) model and treatment

Experimental myocardial infarction was induced in 8-week-old Sprague Dawley rats(270±10g) obtained from ORIENT BIO(Seongnam, Korea), as previously described. Rats were anesthetized by intraperitoneal injection of Zoletil(30mg/kg) and Rompun(10mg/kg). After rats were anesthetized, they were ventilated with positive pressure(180ml/min) using Ventilator(Harvard Apparatus). And then, the rat heart was exposed through incision at the left costal rib. The left anterior descending(LAD) artery was ligated with 6-0 prolene(ETHICON) for 1 hour. After that time, followed by reperfusion and immediately proceeded with injection of cell or PBS. 300ul of PBS or control MSC($1x10^6$ cells in 200ul PBS) or transfected MSC by pEF1/Hisc C ::PSGL-1::EGFP($1x10^6$ cells in 200ul PBS) were injected into myocardium using 31-gauge insulin needle(Becton, Dickinson and Company).

Animal Groups were divided into three groups : PBS injection(LAD ligation and reperfusion), control MSCs(LAD ligation and reperfusion, $1x10^6$ cells), pEF1/Hisc C ::PSGL-1::EGFP-MSCs(LAD ligation and reperfusion, $1x10^6$ cells). 3 weeks after transplantation, the animals were re-anesthetized and sacrificed for MSCs localization and histological study, respectively.

2.4 MSC localization in ischemic sites

To analyze MSCs engraftment(or localization) within infarcted myocardium following experimentally induced rat myocarial infarction, MSCs treated animals were sacrificed 3 weeks after transplantation. The heart of rat was perfused with PBS and fixed in 10% formalin solution overnight. And next day, geart tissue was embedded in OCT compound(SAKURA) and frozen with dry ice, and sectioned transversally with cryostat. Cryosections were mounted with mounting solution containing 4',6-diamidino-2-phenylindole(DAPI)(Santa cruz Biotechnology, Inc) and covered with cover slide. Mounted sections were examined under confocal fluorescent microscope, and DAPI and EGFP were detected in heart sections.

2.5 2,3,5-Triphenyltetrazolium chloride(TTC) staining

Myocardial infarct size was measured by using TTC(sigma-Aldrich, cat no.298-96-4) staining. PBS or MSCs treated animals were sacrificed 4 days atfer implanation. Heart was perfused with PBS and incubated in 1% TTC for 15 minutes at 37°C water bath. And then, heart tissue were fixed in 10% formalin at 4°C for overnight. This heart was sectioned transversally and photographed with digital camera. The infarct size was measured by calculating the ratio of cumulative infarcted area to the entire left ventricle. Analysis of the infarcted area was performed using Image J software program.

2.6 Trichrome staining

Heart was perfused with PBS and fixed in 10% formalin solution. Heart section were stained with Trichrome stain (Masson) kit (Sigma-Aldrich) according to the manufacturer's recommendations. In briefly, the cryosection slides were stained with Bouin's solution at 56°C for 15min and washed in running tap water until yellow color from section was disappeared. And then, slides were stained with Weigert's iron hematoxylin solution for 15min and washed under tap water for 5min. And the sections were stained with Blebrich scarlet-acid fucshin solution for 5min and rinsed in deionized water. Next, the sections were placed in working Phosphotungsitic/Phosphomolybdic acid solution for 5min and Anilline blue solution for 5min. The sections were treated with 1% acetic acid for 2min and rinsed deionized water. The sections were dehydrated through from 70% alcohol to 100% alcohol, clear in xylene. And then, the sections were mounted using permount(Fisher Scientific)

2.7 Immunofluorescence(IF)

To confirm that PSGL-1 expression from MSCs transfected with pEF1/Hisc C ::PSGL-1::EGFP in ischemic heart. MSCs treated animals were 2 weeks after transplantation. The heart or rat was perfused with PBS and fixed in 10% formalin solution overnight. And next day, heart tissue was embedded in OCT compound(SAKURA) and frozen with dry ice, and sectioned transversally with cryostat. Cryosections wre washed PBS containing Triton X-100(Sigma). And sections were permeabilized

with mix of 100% methanol and 30% H_2O_2. Then, washed 3 times with PBS and incubated whit 3% BSA(blocking step). Next, cryosections were incubated with primary antibody(PSGL-1 antibody, Santa cruz Biotechnology, Inc, 1:50) in blocking solution at 4°C for overnight. And then, the sections were washed with PBS Three times and incubated with seconary antibody(Cy5.5 Donkey anti-goat antibody. Biolegned, 1:200) at RT for 1hr. sections were washed with PBS, mounted with mounting solution containing 4',6-diamidino-2-phenyliondole(DAPI)(Santa cruz Biotechnology, Inc) and coverd with cover slide. Section were examined under Olympus fluorescence microscope, and DAPI and Cy5.5 were detected in heart section.

2.8 Echocardiogram

The timepoints is before myocardial infarction(MI), 4days after MI and 21days after MI. Rats were anesthetized by isoflurane chamber and move on array with nose cone for maintain anesthetizing. Thorax hair removed by depilatory cream applying. Rats were maintained at 37 °C via heating pad and rectal probe and were monitored using surface ECG limb electrodes throughout imaging. Transthoracic echocardiography was performed using the Vevo 2100 imaging systems (FUJIFILM VisualSonics, Toronto, Canada) with a probe for rats. Heart rate was checked between 300~400 bpm while imaging by adjusting isoflurane concentration. M-mode and B-mode images of the heart were obtained, and measured ejection fraction, fractional shortening, left ventricle diameter and volume at diastole by this image.

2.9 Statistical analysis

Data are presented as mean ± standard deviation The data was analyzed using multiple T-test followed by Mann-Whitney test. A p-value less than 0.05 were considered to conform statistical significance. All statistical analysis were performed using GraphPad Prism 8 for Windows(version 8.4.2, GrapgPad Software)

Ⅲ RESULTS

3.1 Engraftment of pEF1/Hisc C ::PSGL-1::EGFP transfected MSCs in ischemic heart

MSC surviving is important to improve therapeutic effects in myocardial infarction. Increased MSC surviving can more secrete paracrine factors, so that lead to improve injured myocardium[13]. Modified MSC generally demonstrate more surviving than normal MSC in myocardial infarction[14]. This also conform fluorescence image by stain MSC[15].

After the MSC was injected into the heart, Immunofluorescence(IF) was performed to conform MSC survival. Cardiomyocytes were labeled with dapi, and MSCs were labeled with c-kit(Figure 2A). Counting stained MSCs, MSC transfected with PSGL-1 remained in the myocardium more than twice that of the normal MSC(Figure 2B).

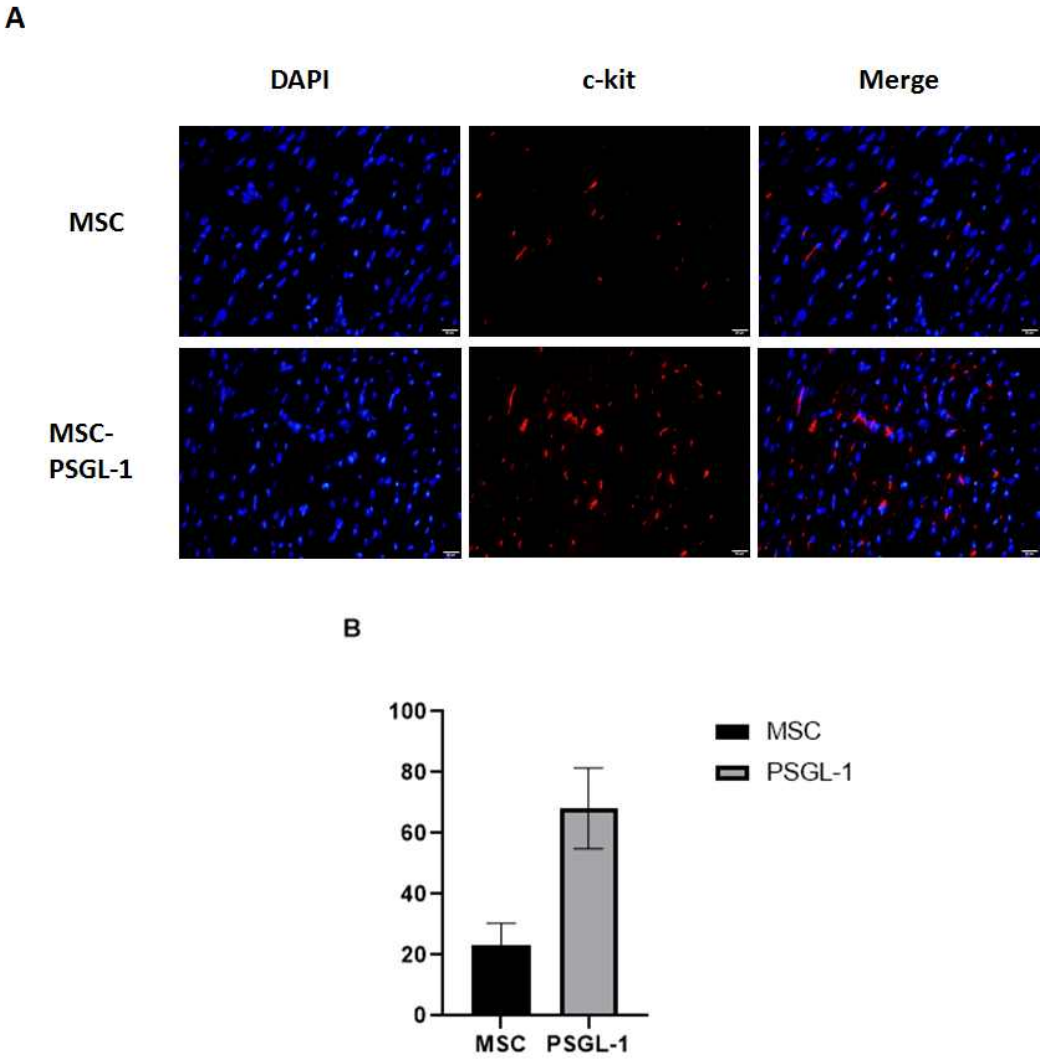
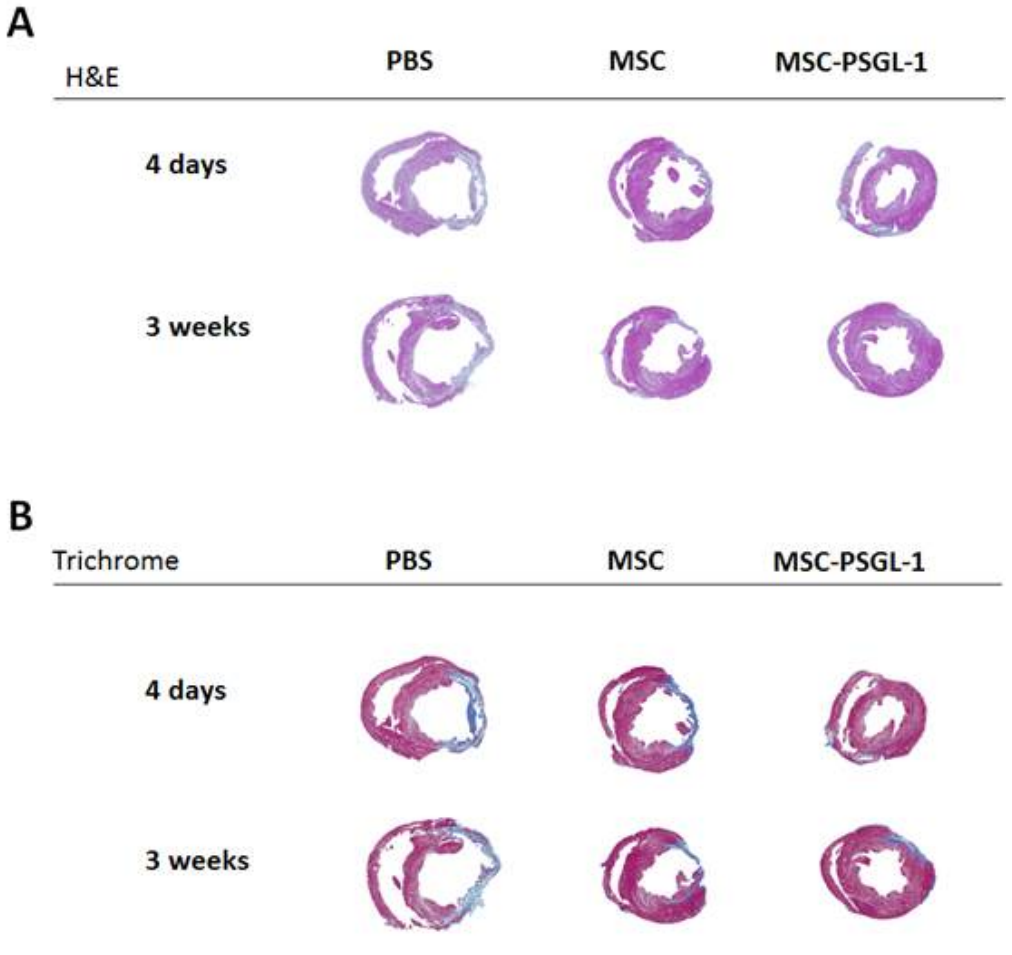


Figure 2. The surviving of MSCs transfected with pEF1/Hisc C ::PSGL-1::EGFP in ischemic heart. The red fluorescence indicated c-kit distribution in ischemic left ventricle. Fluorescence imaging showed more red fluirescence in MSC-PSGL-1 group compared to normal MSC group.(A) The graph quantifying surviving MSCs in ischemic heart by cell couting.(B)

3.2 Enhanced myocardial repair in ischemic myocardium with pEF1/Hisc C ::PSGL-1::EGFP

H&E(Haemotoxylin and Eosin), Masson's trichrome, and TTC (2,3,5-Triphenyltetrazolium chloride) staining are the preferred method for measure at the size of the infarct area[16]. In the H&E, Masson's trichrome staining, the infarct area is stained blue, and in TTC staining, all parts other than the infarction area are stained red. With these methods, the infarct area can be measured as a percentage of the whole myocardium, and the infarct area after treatment can be compared with the normal group. The lower the infarct ratio compared to the normal group, the better the repair was done[16][17].

To conform the therapeutic effects by PSGL-1 transfected MSCs in myocardial infarction, Masson's trichrome and H&E staining were performed(Figure 3A,B). The fibrosis area was measured using Image J software program and the results are as follows: PBS 25.92%, normal MSCs 16.24%, PSGL-1-MSCs 10.66% in 3 weeks later from myocardial infarction(Figure 3C). The fibrosis area was significantly decreased in PSGL-1-MSC group compare to other groups.



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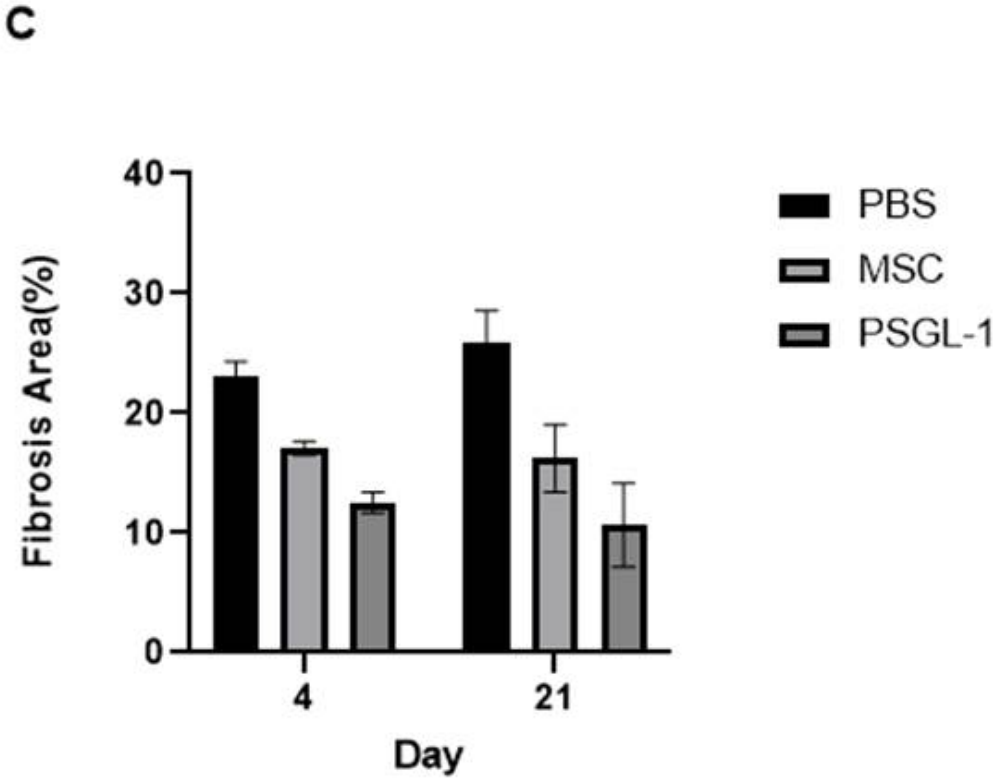
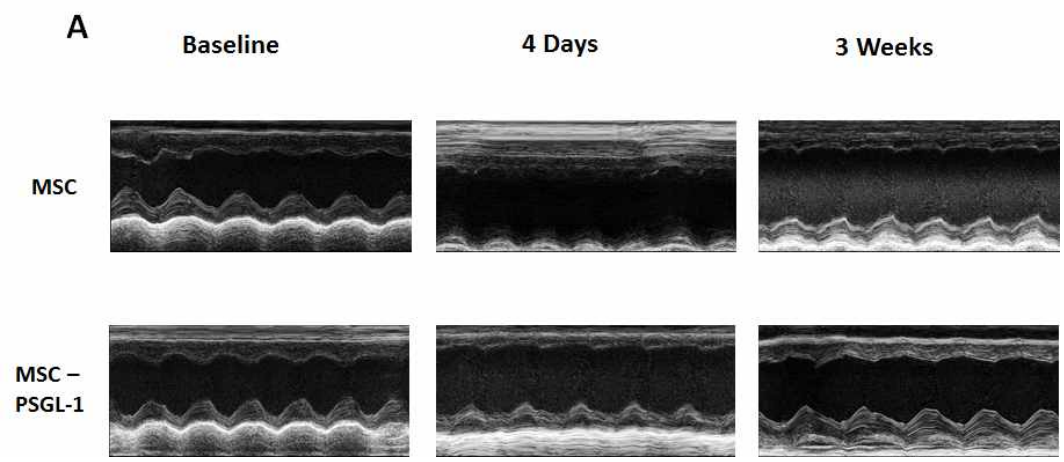


Figure 3. Analysis of histological evaluation. The death cardiomyocytes were not stained red color by H&E staining(A). The fibrosis area was stained blue by Masson's trichrome staining(B). The fibrosis area was quantified by Image J analysis(C). The fibrosis area was significantly decreased in PSGL-1-MSC group compared to other groups.

3.3 Cardiac function imprved by the pEF1/Hisc C ::PSGL-1::EGFP-MSC implantation

Echocardiography is often used to measure heart function along with MRI(Magenetic Resonance Imaging)[18]. Representative echocardiograms for echocardiography are ejection fraction(EF) and fractional shortening(FS). Since both ejection fraction and fractional shortening result in a decrease in cardiac function due to MI, the level of ejection fraction and fractional shortening in the treatment group is higher than that in the normal group, demonstrate more repair than PBS group[19].

Cardiac function was also improved by PSGL-1 transfected MSC implantation. To further investigate cardiac function recovery of ischemic myocardium 3weeks after infarction, transthoracic echocardiographic assessment was performed(Figure 4). The implantation of PSGL-1 transfected MSC increased ejection fraction and fractional shortening(Figure 4B,C). However there is no significant difference in left ventricular diameter and volume at end diastole between all groups(Figure 4C,D).



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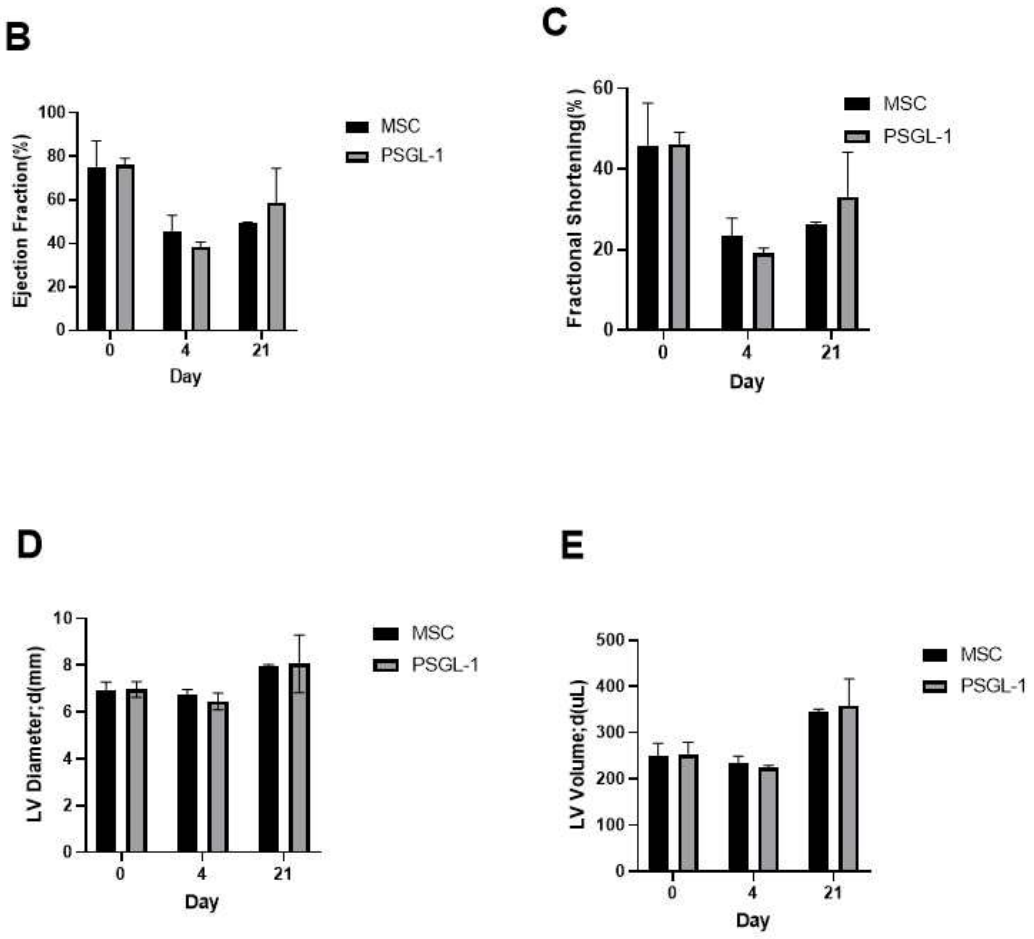

Figure 4. The Implantation of PSGL-1 transfected MSC improved cardiac function. Quantitative analysis of ehcocardiographic data for evaluation of heart functions and left ventricular size after 3weeks from myocardial infarction. (B) Ejection fraction, (C) fractional shortening, (D) left ventricular diameter at end diastole and (E) left ventricular volume at end diastole were assessed.

Ⅳ DISCUSSION

The cardiac ischemia or myocardial infarction was commonly known as a heart disease. An ischemia is the condition that one of arteries or branches in heart becomes blocked suddenly, so heart is starved of oxygen[20]. The Myocardial infarction causes the death of patient due to in necrosis of cardiomyocytes. This is reason for that myocardial infarction was known a major cause of death worldwide[2][21]. The infarcted heart causes cardiac cell death, scar formation, wall thinning and collagen degradation[21][22]. Therefore, many researchers performed to repair the damaged cardiac function and prevent the destructive ventricular remodeling. Recently, various strategies have been suggested to overcome the limitation of stem cell therapy.

This study focused on that one of the cell therapy limitation cell surviving. It was confirmed that the treatment effect increases as cell surviving increases[23]. In infarcted heart, expression of P-selectin is increased[9][24]. And P-selectin bind P-selectin glycoprotein ligand-1(PSGL-1), so we use this. Transfected MSC with PSGL-1 injected myocardial infarction boarder zone and demonstrated MSC surviving increase by immunofluorescence(IF) staining(Figure 2).

Transfected MSC with PSGL-1 also improve heart function. When in myocardial infarction, cardiac function parameters such as ejection fraction and fractional shortening were remarkably decreased[25]. But modified MSC is implanted, many cardiac function indicators recovered(Figure 4). And also decreased infarct size, confirmed H&E, TTC and Masson's

trichrome staining(Figure3). These improving heart repair are due to paracrine fatores secreted by MSCs[26]. Therefore, further studying of this mechanism could enhance the therapeutic effect by MSC therapy.

V CONCLUSION

More cells remained in the myocardium when transfected MSCs with PSGL-1 were injected than normal MSC. So, infarct size and fibrosis have been reduced. And also confirmed the Improvement of heart function by Echocardiography. In short, transfected MSCs with PSGL-1 improve MSC surviving in myocardial infarction and improve therapeutic effects.

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ABSTRACT(In Korean)

심근경색 모델에서의 PSGL-1으로 형질전환한 중간엽 줄기세포의 생착률과 치료효과 개선

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중간엽줄기세포는 많은 임상분야에서 치료제로 쓰이고 있다. 또한 이러한 줄기세포치료는 심근경색이나 심부전 같은 심장질환에서 이용하고 있다. 기존의 줄기 세포 치료법에서 흔히 발생하는 문제는 주입 된 줄기 세포가 병변부위로 제대로 이동하지 않고 다른 장기에 분산되어 퍼진다는 것이다. 실제로 혈관에 주입 된 줄기 세포가 병변 부위로 이동하는 효율은 2 % 미만이며 나머지 세포는 주로 폐 또는 신장에서 발견되고 이마저도 단기간(4주 이내) 내에 사라지는 것으로 보고되었다. MSC의 이러한 낮은 생착률을 증가시키기 위해, 본 연구에서는 초기 면역 반응 동안 혈관에서 순환하는 면역 세포가 염증 부위로 이동하는 기작에 관여하는 단백질을 줄기 세포에 적용하였다. 세포 괴사와 같은 조직 손상이 발생하면, 인터류킨과 같은 신호 물질이 관련 부위로부터 분비되어 혈관 내피 세포를 자극하고, 셀렉틴 및 CAM과 같은 단백질이 표면에 발현된다. 면역 세포의 표면에서 발현되는 P- 셀렉틴 당 단백질 리간드 -1 (PSGL-1) 또는 L- 셀렉틴은 혈관 내피 세포의 자극에 의해 발현되는 단백질에 결합하며, 혈관 내 이동하는 줄기 세포의 속도를 늦춰 병변부위의 혈관 안으로 들어오는 세포의 양을 증가시킬 수 있다. 우리는 이 원리를 이용하여, 유전자 형질도입을 통해 중간엽줄기세포 표면에 PSGL-1을 발현시키고 치료효과를 증가시켰다.

유전자 형질도입은 바이러스성이나 비 바이러스성 운반체를 통해 진행할 수 있다. 우리의 최종 목표 이 치료법을 임상에 적용하는 것이기 때문에 비 바이러스성 운반체인 ABP를 통하여 PSGL-1을 형질도입 하였다. 그 후, PSGL-1을 발현하는 MSC를 심근 경색 랫드 모델에 주사하고 각 기간 및 단계에서 유전자 도입 줄기 세포의 효과를 검증 하였다. 심근 경색 모델을 만들고 줄기 세포를 주입 하여 3주 후에 심장 조직을 얻어, 경색 부위에 남아있는 MSC를 측정하기 위해 면역 형광 염색을 수행 하였다. 또한 트라이크롬 염색과 TTC

염색, H&E염색을 통해 경색률과 석화화 비율이 감소된 것을 확인하였다. 마지막으로 심초음파를 확인하여 심장 기능의 개선을 확인하였다.

핵심 되는 말 : P-셀렉틴 당단백질 리간드, 줄기세포치료법, 세포 유전자도입, 세포 이식, 심근경색