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Review Article

Endothelial Regeneration in Repair from Vascular Injury and Stem Cell Biology

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Summary

In the process of repair from tissue injury, 'regeneration' naturally occurs in-vivo. Also in the vessels, mechanical stress induces injury, but then the injured sites are repaired by tissue regeneration. Our research has long focused on stem cell biology in vascular injury and repair. In the process of repair from vascular injury, bone marrow-derived stem cells including endothelial progenitor cells (EPCs) are recruited, and the EPCs differentiate into endothelial cells, leading to endothelial regeneration. On the other hand, also in the cardiovascular scene, regenerative medicine has been clinically applied as vascular and myocardial regeneration. We have been promoting vascular regeneration therapy (angiogenesis therapy) for critical limb ischemia, using adipose-derived regenerative (stem) cells (ADRCs). Now, we are planning a new project for research and clinical application of regenerative medicine, using the multi-lineage differentiating stress enduring (Muse) cell as a novel cell source.

Key Words: atherosclerosis, endothelial regeneration, angiogenesis, adipose-derived regenerative cells, multi-lineage differentiating stress enduring cell

Introduction

In the process of repair from tissue injury, 'regeneration' naturally occurs in-vivo. The natural regeneration includes regeneration of shedded skin after burns, healing of sutures after surgery and healing after bone fracture. Also in the vessels, mechanical stress induces injury, but then the injured sites are repaired by tissue regeneration. Since Russel Ross[1] proposed 'response-to-injury hypothesis' in 1976, its details have been elucidated in the 2000s. Following impairment of endothelial function by risk factors such as hypertension, diabetes mellitus, dyslipidemia, etc., inflammation and oxidative stress produce vascular injury and then repair mechanism occurs against it. When balance between vascular injury and repair leans toward injury, atherosclerosis would develop.

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Inflammation and atherosclerosis

A representative in-vivo animal model for atherosclerosis is the balloon-injury vascular model. On the

other hand, we believe that percutaneous coronary intervention (PCI) is a powerful in-vivo human model for atherosclerosis research, and focused on pathophysiology of vascular injury after PCI, especially inflammatory reaction. It is known that an adhesion molecule, leukocyte integrin Mac-1 (CD11b/CD18) plays an essential role in the inflammation at the site of vascular injury. Animal models of Mac-1 blockage by antibody[2] or Mac-1 knock-out models[3] showed that neointimal thickening after balloon-induced vascular injury was suppressed. We observed serial change in the expression of Mac-1 on the surface of neutrophils in patients undergoing PCI, using the flow cytometric analysis. As a result, Mac-1 was upregulated and activated maximally at 48-hr post-PCI, being associated with development of restenosis[4-6]. The result suggests that Mac-1 is a key player in the mechanism of atherosclerosis progression also in human.

Repair mechanism of injured-vessel

It has been suggested that bone marrow-derived stem cells play a crucial role in the process of repair from vascular injury after PCI. When vessel wall is injured by balloon or stent in the PCI procedure, vascular endothelial cells are shedded. Thereafter, strong inflammatory reaction occurs at the injured-vessel site, and then triggered by the inflammation, bone marrow-derived stem cells are mobilized and recruited to the injured-vessel site. The bone marrow-derived stem cells include both endothelial progenitor cells (EPC), and smooth muscle progenitor cells (SMPC). The EPCs differentiate into vascular endothelial cells, leading to endothelial regeneration (reendothelialization) and neointima formation, which results in stent coverage by neointima. This is a physiological repair reaction process. On the other hand, the SMPCs differentiate into vascular smooth muscle cells, which proliferate, also playing some role on neointima formation. However, overgrowth of the smooth muscle cells causes restenosis. Thus, the restenosis is an overreaction in the vascular repair process. Formerly, the restenosis was the biggest weakness in the PCI scene. Since drug-eluting stents (DESs) was developed, its frequency has significantly decreased. However, a DES-specific novel problem has surfaced. Main mechanism for restenosis prevention of the DES is a direct inhibition of smooth muscle cell proliferation by drugs, such as immune suppressants, installed on the stent surface. The other mechanism includes strong anti-inflammatory effects by the installed drugs, which result to inhibit the differentiation of SMPCs into vascular smooth muscle cells. However, DESs simultaneously inhibit the differentiation of EPCs into vascular endothelial cells, resulting in inhibition of reendothelialization, neointima formation and neointimal stent coverage. Therefore, DESs suppress not only the overreaction in vascular repair but also the physiological repair reaction. In other words, DESs is a double-edged sword[7].

We serially observed mobilization of CD34+ bone marrow-derived stem cells in patients undergoing PCI, using the flow cytometry. The number of CD34+ cells, including both EPCs and SMPCs, in peripheral blood, increased maximally on the day 7 post-PCI in patients undergoing implantation of bare metal stents (BMSs), compared with baseline pre-PCI. Especially, the increase in CD34+ cells was more prominent in patients who developed restenosis, compared with those who did not. In contrast, the number of CD34+ cell in the peripheral blood decreased at the day 7 post-PCI in patients, compared with baseline pre-PCI, in whom first generation DES, sirolimus eluting stent (SES) was implanted. In addition, we isolated circulating stem cells from the patients on the day 7 post-PCI, and cultured them in both endothelial cell medium and smooth muscle cell medium. Consequently, the cell culture in the endothelial cell medium resulted in the differentiation of stem cells into CD31-positive endothelial-like cells in patients undergoing BMS implantation. The cell culture in the smooth muscle medium resulted in the differentiation of stem cells into α-smooth muscle actin (α-SMA)-positive smooth muscle-like cells in patients undergoing BMS implantation, especially in patients who experienced restenosis. In contrast, these cell differentiations were substantially inhibited in patients undergoing SES implantation (Fig. 1)[7,8]. In our subsequent research, the mobilization of EPCs (CD34+/CD133+/CD45low cells) was inhibited more strongly by implantation of second generation DESs, i.e., zotarolimus eluting stent (ZES) and everolimus eluting stent (EES), compared with BMS, though not as much as SES[9]. Third generation DESs, in which biodegradable poly-

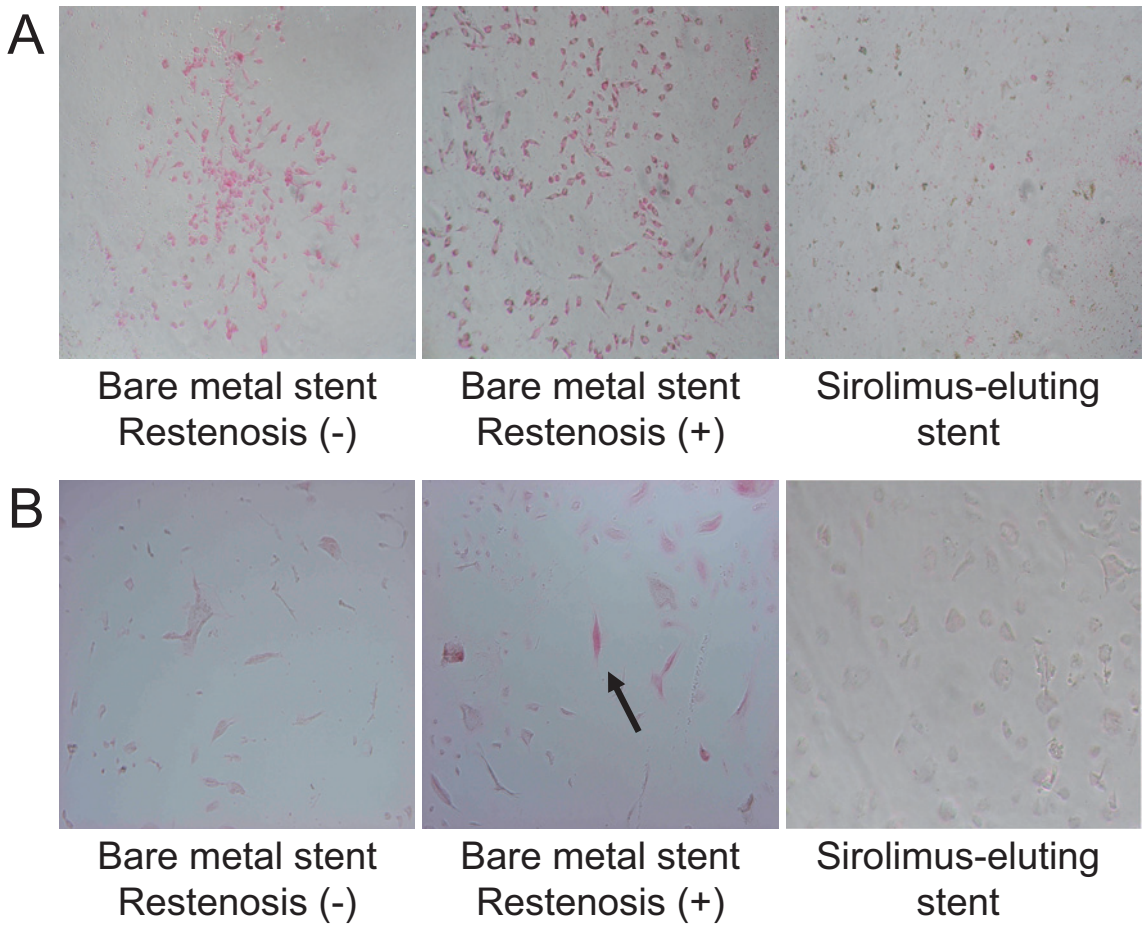

Figure 1 Differentiation of cultured circulation stem cell from the patients on the day 7 after coronary stent implantation. (A) The cell culture in endothelial cell medium resulted that the stem cells differentiated into CD31-positive endothelial-like cells in patients undergoing bare metal stent implantation, both patients who experienced restenosis and those who did not. In contrast, the cell differentiations were substantially inhibited in patients undergoing sirolimus-eluting stent implantation. (B) The cell culture in smooth muscle medium resulted that the stem cells differentiated also into α-smooth muscle actin-positive smooth muscle-like cells in patients undergoing bare metal stent implantation, especially in patients who experienced restenosis (arrow). In contrast, the cell differentiations were also substantially inhibited in patients undergoing sirolimus-eluting stent implantation.

mer is coated on the stent surface to install the drugs, have been devised for early vascular repair in stent-injured vessel sites. We observed that inhibitory effects of third generation DESs on mobilization of EPCs (CD34+/KDR+ cells) was milder, compared with second generation DESs. Hereafter, the development of new generation DESs would be accompanied by a paradigm shift as 'from reduction of restenosis to induction of physiological vascular repair'.

Regenerative medicine, regenerative medical research

In a broad sense, the research for mechanism of endothelial regeneration such as bone marrow-derived stem cells in the process of repair from PCI-induced vascular injury is included in regenerative medical research. And the therapeutic approach to induce the endothelial regeneration and physiological vascular repair is included in regenerative medicine. In general, however, regenerative medicine refers to the treatment for reconstruction of the tissue/organ function by implantation of somatic stem cells, i.e., the cell therapy. Also in the field of cardiology, regenerative medicine has been applied to target vascular regeneration and myocardial regeneration. Especially, an angiogenesis therapy by implantation of bone marrow-derived stem cells (CD34+ cells) or peripheral blood mononuclear cells has been performed for critical limb ische-

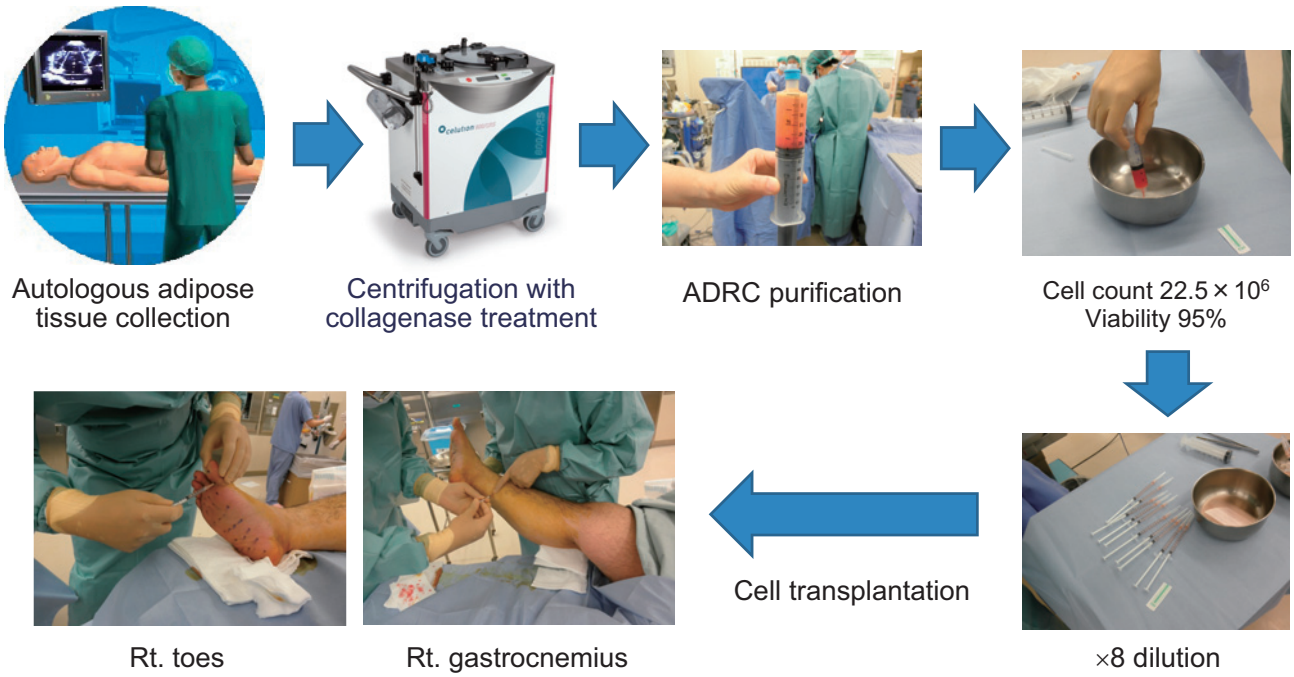

Figure 2 Cell transplantation using adipose-derived regeneration cells (ADRCs) for critical limb ischemia.
The ADRCs were purified from abdominal subcutaneous adipose tissue and transplanted cells into right gastrocnemius muscles, right gastrocnemius and right toes by injection.

mia in peripheral artery disease.

Formerly, adipose tissue was considered to be a mere energy storage. However, it has been elucidated to be an endocrine organ, which synthesizes various physiologically active substances, and to include pluripotent stem cells. Although most of the volume of adipose tissue is occupied by adipocytes, its interstitium contains many cells including mesenchymal stem cells (MSCs), EPCs, endothelial cells, pericytes and macrophages[10]. Cell fraction of these cells can be isolated by collagenase treatment of adipose tissue and following centrifugation. The cell fraction, purified and collected from aspirated abdominal subcutaneous fat by a special centrifuge, has excellent tissue regeneration ability. And such the fraction is called the adipose-derived regenerative cells (ADRCs) and applied for regenerative medicine in clinical settings of various fields. The angiogenic effect of ADRCs has been shown in animal limb ischemia models and its molecular mechanism is being elucidated[11]. We have conducted vascular regeneration therapy, i.e., angiogenesis therapy, for 5 patients with critical limb ischemia, using the ADRCs, as a clinical trial at 8 facilities nationwide[12].

Here we present a case of 45-yr male with critical limb ischemia caused by Buerger's disease, who underwent regeneration therapy using ADRCs. We purified ADRCs from abdominal subcutaneous adipose tissue and transplanted the cells into right gastrocnemius muscles, and hypodermis of right toes by injection (Fig. 2). As a result, the erosion of the right third toe improved, right foot pain was significantly relieved and 6 minutes walking distance increased. And, substantial angiogenesis was observed in angiography (Fig. 3). The mechanism of angiogenesis by ADRCs as well as bone marrow-derived stem cells is that these cells do not differentiate directly into vascular cells, but angiogenesis is caused by stimulation of various cytokines produced by the cells, the paracrine effect[13].

One of the important factors on effects of regeneration therapy by stem cell implantation is cell viability. We have been conducting efforts to improve the viability of regeneration cells. We have reported that micro-/nano-particles produced by mixture of low-molecular weight heparin and protamine improved cell viability and angiogenetic effects if adding to ADRCs[14]. In addition, we found that manipulations of injury and ischemia on adipose tissue by mincing adipose parenchyma and ligating the subcutaneous fat feeding ar-

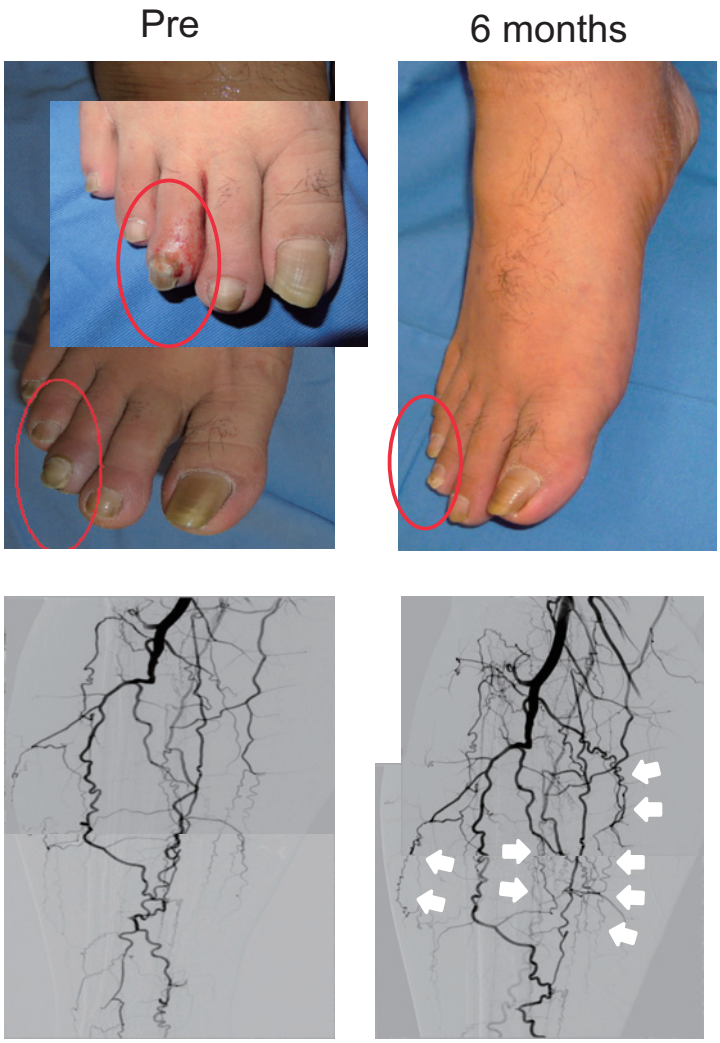

Figure 3 Representative case of 45-yr male with clitical limb-ischemia caused by Buerger disease.
Six months after implantation of ADRCs, erosion in the right third toe improved, angiography showed substantial angiogenesis (arrows). Right foot pain drastically decreased and 6 minutes walking distance increased.

tery improved cell viability and angiogenetic effects for isolated ADRCs, and we called such effect of manipulations as the "priming" effect[15].

New paradigm for regenerative medicine

We are now focusing on the "multi-lineage differentiating stress enduring (Muse) cell" as a novel cell source in our next project for regenerative medicine. Muse cell is an endogenous non-tumorigenic pluripotent cell. In case of tissue injury, receiving an injury alert signal of sphingosine-1-phosphate (S1P), Muse cells mobilize from bone marrow, adipose tissue, etc., accumulate to damaged tissue, and spontaneously differentiate into tissue-compatible cells after homing.[16]. Although Muse cells also have cytokine-producing ability[17], the tissue regeneration by the Muse cells is con-

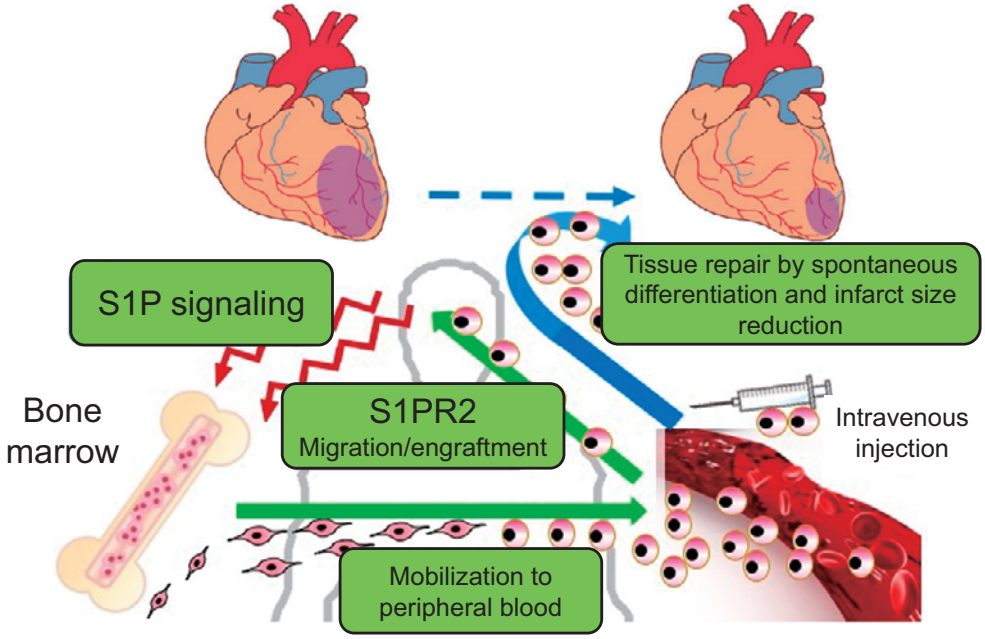

Figure 4 Concept of Muse cell therapy for acute myocardial infarction.
When myocardial infarction develops, in response to tissue damage, an alarm signal, sphingosine-1-phosphate (S1P), is released. In response to S1P, Muse cells are mobilized from the bone marrow, etc., into the peripheral blood via S1P receptor 2 (S1PR2), and then migrate and engraft in the injured tissue, resulting in tissue repair by spontaneous differentiation. Muse cell therapy is a treatment method that enhances tissue repair ability by replenishing donor cells.

sidered to be based on their own differentiation rather than paracrine effects. Muse cells are identified as cells positive for stage-specific embryonic antigen-3 (SSEA-3), a surface marker for pluripotent stem cells, by using flow cytometric analysis[18]. In case of cell therapy, Muse cells do not require the introduction of new genes, nor differentiation prior to transplantation, unlike induced pluripotent stem (iPS) cells. Cells from the patient themselves are not required, i.e., allogenic transplantation of cells from donors is possible, because there is no rejection. Since Muse cells have the property of spontaneously homing to the damaged tissue, intravenous administration can be used for treatment. In addition, Muse cells are no tumorigenic, so there is no concern about development of cancer after the treatment. Therefore, Muse cell treatment can be performed through an intravenous drip of donor-derived cells, and there are no ethical complications[19]. It has been observed that Muse cells are mobilized in patients with acute myocardial infarction at acute phase[20] and that intravenous injection of donor-derived Muse cells considerably reduce infarct size and improve cardiac function in rabbit myocardial infarction model[21]. Therefore, a clinical trial of Muse cell treatment for acute myocardial infarction is now ongoing and we have also participated in the trial. The concept of Muse cell treatment for acute myocardial infarction is shown in Fig. 4. In the event such as myocardial infarction, an alarm signal, sphingosine-1-phosphate (S1P) is released. In response to this, Muse cells are mobilized from bone marrow into peripheral circulation, and further migrates to the infarct area, engrafts, and spontaneously differentiates into myocardial cells, leading to tissue repair. Muse cell therapy is a treatment that enhances tissue repair by replenishing donor cells.

In addition to the clinical trial, we have started our own Muse cell research, targeting acute myocardial infarction and acute myocarditis.

Muse cell research project for acute myocardial infarction

Tanaka et al.[18] measured the number of Muse cells in the peripheral blood of patients with acute myocardial infarction on the days 0, 1, 7, 14, and 21 after the onset. As a result, compared with baseline on the day 0, Muse cell number increased with the peak of 1.5-fold

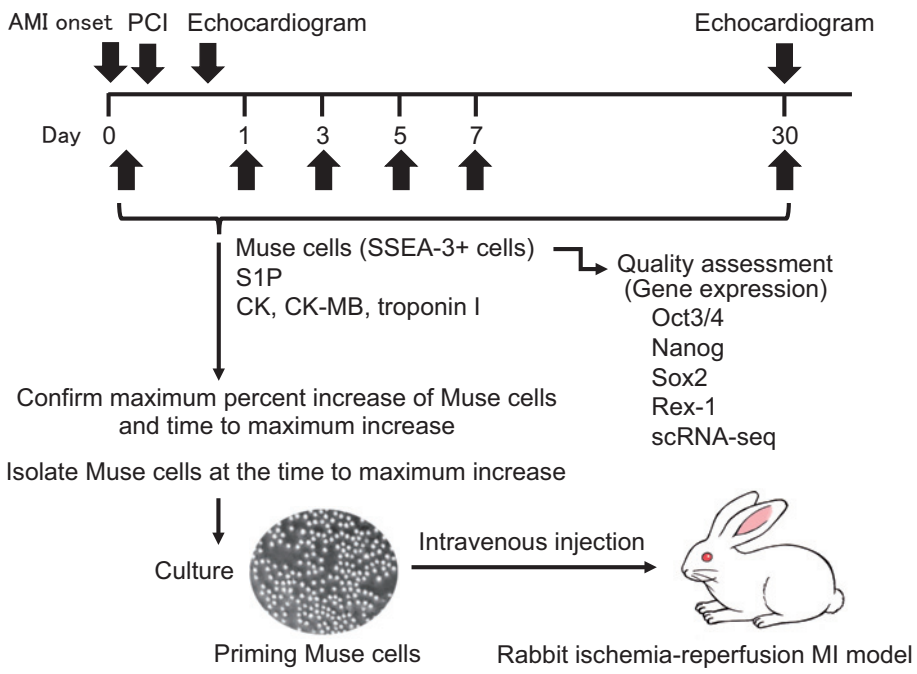

Figure 5 Muse cell research project for acute myocardial infarction.

We measure the number of Muse cells on the days 0, 1, 3, 5, 7 and 30 after the onset of acute myocardial infarction. Then we assess regenerative quality of the Muse cells isolated on the day 3 and 5, the number of which shows peak, by observation of expression of pluripotency genes such as Oct3/4, Nanog, Sox2 and Rex-1 and by non-targeted gene analysis using the single-cell RNA sequencing (scRNA-seq). In addition, we examine the association between the number and quality of patients' Muse cells and cardiac function at chronic phase. Finally, we intravenously injected Muse cells isolated from patents at the time point of peak cell number, i.e., priming Muse cells, into rabbit ischemia-reperfusion myocardial infarction model, and compare the infarct size and cardiac function with non-priming cells.

AMI, acute myocardial infarction; PCI, percutaneous coronary intervention; SSEA-3, stage-specific embryonic antigen-3; S1P, sphingosine-1-phosphate; CK, creatin kinase; scRNA-seq, single-cell RNA sequencing

increase on the day 1, and had gradually decreased on the day 21. It is, however, hypothesized that the peak of Muse cell number would be shown between the day 1 and 7, on which the cell number was not measured in the study. Therefore, we just started to measure the number of Muse cells on the days 0, 1, 3, 5, 7 and 30 after the onset of acute myocardial infarction. Hereafter, we are planning following research projects. First, we assess regenerative quality of the Muse cells isolated from acute myocardial infarction patients on the day when the cell number shows peak, by observation of expression of pluripotency genes such as Oct3/4, Nanog, Sox2 and Rex-1 and by non-targeted gene analysis using the single-cell RNA sequencing (scRNA-seq). Next, we assess association between the number and quality of patients' Muse cells and cardiac function at chronic phase. Finally, we supply (intravenously inject) Muse cells isolated from patients at the time point of peak cell number, i.e. "priming" Muse cells, into rabbit myocardial infarction models and compare the infarct size and cardiac function with "non-priming" Muse cells, since human Muse cells can be used for the xenotransplantation to rabbits without rejection (Fig. 5).

Muse cell research project for acute myocarditis

Acute myocarditis is an inflammatory disease of the heart muscle (myocardium), mostly caused by a viral infection. Other causes include bacterial infections, certain medications, toxins, and autoimmune disorders. Myocarditis is often associated with pericarditis. In most cases, patients are asymptomatic or have mild to moderate heart failure symptoms. However, in rare cases, myocarditis rapidly becomes severe and mortal, which is called fulminant myocarditis[22].

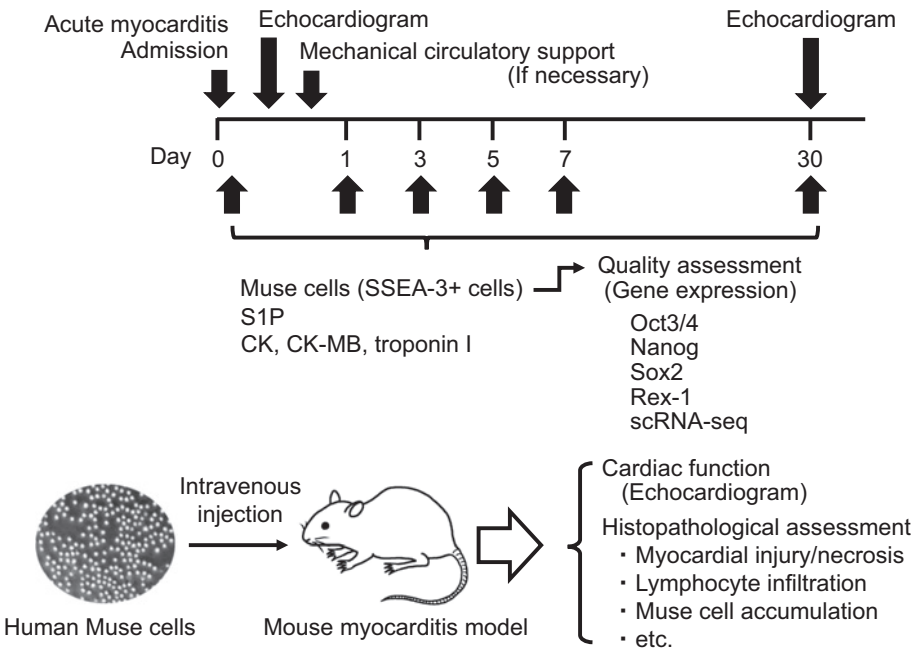

Figure 6 Muse cell research project for acute myocarditis.
We measure circulating Muse cells serially after admission in patients with acute myocarditis and analyze gene expression of Muse cells isolated from patients to assess regenerative and reparative ability. We intravenously inject Human Muse cells into mouse myocarditis model established by viral infection, drug induction or immunomodulation and examine the effects of Muse cell therapy for prevention of fulminant disease.

We serially measure circulating Muse cells also in patients with acute myocarditis, and perform gene analysis similarly to the acute myocardial infarction project, and elucidate pathophysiological significance of endogenous Muse cells on fulminant myocarditis. In addition, since human Muse cells can be also used for the xenotransplantation to mice, we intravenously inject them into mouse myocarditis model established by viral infection, drug induction or immunomodulation and examine the effects of Muse cell therapy for prevention of fulminant disease (Fig. 6).

Expectation for regeneration medicine using Muse cells

Since Muse cells have the property of spontaneously migrating to the injured site, intravenous injection is available for Muse cell treatment. Since Muse cells have low telomerase activity, the concern about canceration is extremely low. Furthermore, since there is no rejection reaction, allogeneic cells may be used. Because of these unique properties, Muse cells can be used for treatment very easily and safely, as if they were blood derivatives. Currently, in addition to the above-mentioned acute myocardial infarction, clinical trials are being conducted also for cerebral infarction, epidermolysis bullosa, and spinal cord injury[17]. We hope that regenerative therapy using Muse cells will become widespread in clinical practice in the future.

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