Antagomir-92a impregnated gelatin hydrogel microsphere sheet enhances cardiac regeneration after myocardial infarction in rats

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Introduction: We investigated whether attachment of gelatin hydrogel microsphere (GHM) sheet impregnated with antagomir-92a on the infarcted heart promotes angiogenesis and cardiomyogenesis, and improves cardiac function after myocardial infarction (MI) in rats.

Methods: GHM sheet impregnated with antagomir-92a, its scramble sequence antagomir-control sheet or the sheet alone was attached on the area at risk of MI after the left anterior descending coronary artery ligation. Bromodeoxyuridine (BrdU) was included in the sheet to trace proliferating cells.

Results: The antagomir-92a sheet significantly increased capillary density in the infarct border zone 14 days after MI compared to the antagomir-control sheet or the sheet alone, associated with an increase in endothelial cells incorporated with BrdU. The antagomir-92a sheet significantly increased cardiac stem cells incorporated with BrdU 3 days after MI in the infarct border zone. This was associated with an increase in cardiomyocytes incorporated with BrdU 14 days after MI. Scar area was significantly reduced by the antagomir-92a sheet compared to the antagomir-control sheet or the sheet alone (12.8 ± 1.3 vs 25.2 ± 2.2, 24.0 ± 1.7% LV area, respectively) 14 days after MI. LV dilatation was inhibited, and LV wall motion was improved 14 days after MI in rats with the antagomir-92a sheet compared to the antagomir-control sheet or the sheet alone.

Conclusions: These results suggest that attachment of the GHM sheet impregnated with antagomir-92a on the area at risk of MI enhances angiogenesis, promotes cardiomyogenesis, and ameliorates LV function.

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1. Introduction

Since current pharmacological and interventional approaches are limited to salvage the damaged heart after myocardial infarction (MI), cardiac regeneration therapy has emerged as an attractive treatment option for severe MI. However, the ideal method of cardiac regeneration therapy has not been established.

Cardiac regeneration therapy consists of cell-based or molecular-based therapy. Each therapy possesses advantages and disadvantages. Cell-based therapy can directly replenish the damaged heart. However, successful cell transplantation requires optimizing the best cell type and site for engraftment, overcoming limitations to cell migration and tissue integration, and occasionally needing to control immunologic reactivity [1]. Therapeutic approaches for cell-based therapy involve bone marrow-derived mononuclear cells and their subsets such as mesenchymal stem/
stromal cells (MSCs), endothelial progenitor cells as well as adipose tissue-derived MSCs, cardiac tissue-derived stem cells, and cell combinations. Clinical trials employing these cells have demonstrated that cellular therapy is feasible and safe, but overall clinical efficacy in patients with MI is modest [2,3]. Therefore, cell-based therapy is still challenging and further innovations are needed to be established as a common clinical practice.

On the other hand, molecular-based therapy has mainly focused on therapeutic angiogenesis, because angiogenesis is a key to success for cardiac regeneration after MI. Protein delivery or gene transfer of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) has demonstrated improvement in tissue perfusion and morphological and angiographic evidence of new vessel formation in various instances of animal models [4,5]. Such reproducible and credible successes in early animal studies have prompted clinical trials employing angiogenic growth factors. Unfortunately, in spite of promising early data coming from many pioneering clinical trials, recent larger and better-designed clinical trials provided disappointing results. An appreciable number of randomized controlled trials using VEGF and FGF proteins have shown far from optimal results [6]. Similarly, several phase I and phase II clinical trials using angiogenic gene transfer in patients with coronary artery disease have provided limited evidence regarding the efficacy and long-term sustainability of therapeutic effect, although generally supporting the safety and tolerability of angiogenic gene transfer [7]. Such limited success of molecular-based therapy using angiogenic gene transfer for cardiac regeneration may be attributed to the fact that tissue repair is not a simple process. Understanding cells, the signals that they respond to, and the keys to appropriate survival and tissue formation are orders of magnitude more complicated than understanding the pathways targeted by most genes. Thus, it is anticipated that targeting any single genes is difficult to confer appreciably beneficial outcomes for regeneration of the heart after MI.

MicroRNAs (miRs) are small non-coding RNAs with short ~22 nucleotide RNA sequences that bind to complementary sequences in the 3′ UTR of multiple target mRNAs, usually resulting in their silencing or translational repression. Each miR inhibits the function of hundreds of target RNAs, thereby integrating and fine-tuning the network of cellular processes involved in plethora of biological events including cardiovascular development and angiogenesis [8,9]. The ability of single miRs to modulate multiple gene expression makes them an attractive tool in cardiac regeneration therapy.

The miR-17-92 cluster has been considered as a therapeutic target for angiogenesis. The miR-17-92 cluster is composed of miR-17, miR-18a, miR-19a/b, miR-20a, and miR-92a. It was among the first miRs that were linked to tumor angiogenesis [10]. Overexpression of the entire miR-17-92 cluster in myc-induced tumors increased angiogenesis by a paracrine mechanism [11]. Interestingly, miR-92a has been found as a negative regulator of endothelial function and angiogenesis. Bonauer et al. [12] demonstrated that miR-92a is increased in the heart after MI, and systemic administration of antagonim-92a augmented neovascularization and repaired the infarcted tissue in mice. Moreover, the efficacy of anti-miR-92a therapy may not be confined to angiogenesis but is also attributed to cell-protective and anti-inflammatory effects [13]. Such pleiotropic salutary effects of anti-miR-92a on cardiac regeneration have prompted the investigators to develop a delivery system to optimally antagonize miR-92a in the post-infarction myocardium.

A critical issue in systemic administration of pro-angiogenic proteins or genes is unwanted angiogenesis in off-target organs such as cancer tissues and eyes with diabetic retinopathy. Systemic administration also requires a large quantity of drugs. Local delivery of pro-angiogenic agents overcomes such drawbacks. Indeed, catheter-based delivery of locked nucleic acid-modified antisense miR-92a was found to be more effective than systemic delivery in protecting the pig heart after MI [13]. However, proteins, plasmids or oligonucleotides are readily degraded or dissipated after local delivery to the target tissue. Therefore, the therapeutic efficacy of bolus injection of antagonim-92a into the heart may be limited. To achieve long-lasting delivery of antagonim-92a into the infarcted myocardium, we developed a biodegradable gelatin hydrogel microsphere (GHM) sheet that is slowly degraded and release impregnated plasmid DNA or proteins over 14 days [14]. We attempted to use the GHM sheet impregnated with antagonim-92a in the rat with acute MI, because mir-92a increases on the day 1 after MI, peaked on the day 2 and then gradually decreases over 14 days [12]. Therefore, the efficacy of the GHM sheet was thought to be maximally brought out by applying it to the heart immediately after MI. In this acute MI model, we investigated whether controlled local delivery of antagonim-92a to the heart increases angiogenesis, enhances cardiomyogenesis, and improves left ventricular (LV) function after MI.

2. Methods

2.1. Preparation of GHM-impregnated sheet

Antagonim-92a and its scramble sequence antagonim-control were synthesized by Greiner Bio-One (Frickenhausen, Germany) as described previously [12]. The GHM sheet used in the present study was prepared as described previously [15]. Briefly, GHMs were prepared by chemical cross-linking of aqueous gelatin solution with glutaraldehyde (GA). Gelatin with an isoelectric point of 5.0 was prepared as an acidic gelatin. After we mixed 2.5 mmol of aqueous GA solution (Wako Pure Chemical Industries, Osaka, Japan) with aqueous gelatin solution (5 wt%) preheated at 40 °C, the mixed aqueous solution was cast into balance dishes and left for 12 h at 4 °C to allow for chemical cross-linking of gelatin. The resulting GHM sheets were placed in 100 mmol/L aqueous glycine solution and then digested at 37 °C for 1 h to block the residual aldehyde groups of unreacted GA. Cross-linked GHM sheets were thoroughly washed with double-distilled water (DDW), freeze-dried, and sterilized with ethylene oxide gas. GHM sheets were then trimmed in approximately 10 × 10-mm squares and 0.7-mm thick. Next, DDW containing 1 mg of antagonim-92a or antagonim-control was added drop-wise onto freeze-dried GHM sheets under sterile conditions and then left for 1 h to allow the GHM to be impregnated. The RNA-free, empty GHM sheets were prepared by the same method as described above.

2.2. Animal preparation

Male Sprague–Dawley rats weighing 250–300 g were used in the present study. All animals were handled in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of the Care and Use of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care Committee of Kansai Medical University (Moriguchi, Japan).

2.3. Surgical procedures

The rats were anesthetized with xylazine (5 mg/kg, s.c.) and ketamine (100 mg/kg, i.m.), and placed on a temperature-controlled surgical table. The trachea of rats was cannulated with a polyethylene tube connected to a respirator (Shinano, ...
Tokyo, Japan) with a tidal volume set at 2.0 ml and a rate set at 100/min. The rats were then anesthetized with 1.5–2.0% isoflurane under controlled ventilation with a respirator for the remainder of the surgical procedure. A left thoracotomy was performed between 4th and 5th ribs, and the pericardial sac was removed. The left anterior descending (LAD) coronary artery was ligated at a middle portion with 6-0 prolene sutures to produce MI. The rat receiving sham surgery underwent the same procedure except the suture was passed under the coronary artery and then removed. The GHM sheet was cut into a trapezoid shape and fixed on the epicardium surrounding the area at risk of infarction with 3 sutures (Fig. 1). The chest was then closed in three layers (ribs, muscle, and skin), and the animal was allowed to recover. Body temperature was maintained at 37°C throughout the surgical procedure. Buprenorphin (0.1 mg/kg i.p.) was administered after surgery to alleviate pain.

2.4. Echocardiography

Transthoracic echocardiography was performed 1 h and 14 days after the LAD coronary artery ligation using a SONOS-7500 echocardiography system (Philips Medical Systems, Andover, MA, USA) equipped with a 15-MHz transducer. The following parameters were measured: 1) LV wall motion score (1 = normal, 2 = hypokinetic, 3 = akinetic, 4 = dyskinetic, and 5 = aneurismal), 2) maximal LV end-diastolic diameter (LVDd), and 3) fractional shortening (FS) calculated as \[ \text{FS} = \frac{\text{LVDd} - \text{LV end-systolic diameter (LVDs)}}{\text{LVDd}} \times 100. \] Wall motion score index was evaluated as described previously [16]. All measurements were averaged for 3 cardiac cycles and performed by an experienced operator blinded to the treatment group.

2.5. Immunohistochemical analysis

To identify proliferating cells 100 mg/kg of bromodeoxyuridine (BrdU) (Sigma, Tokyo, Japan) was impregnated into the GHM sheet. Three days or 14 days after the LAD coronary artery ligation the rats were sacrificed by intraperitoneal injection with overdose sodium pentobarbital (100 mg/kg). The heart was removed quickly and cut horizontally at the mid-LV level. The frozen sample was sectioned embedded in paraffin, and sectioned at a 6 μm thickness. The section was stained with Masson’s trichrome, and the area at risk of infarction and the scar area were quantified by morphometric analysis as described previously [17].

2.6. Measurement of infarct size

Fourteen days after the LAD coronary artery ligation the heart was cut horizontally at the mid-LV level, fixed with 10% formalin, embedded in paraffin, and sectioned at a 6 μm thickness. The section was stained with Masson’s trichrome, and the area at risk of infarction and the scar area were quantified by morphometric analysis as described previously [17].

2.7. Statistical analysis

Statistical analyses were conducted with a commercially available software package (StatView 5.0, SAS Institute Inc, Cary, NC, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. All numerical data are expressed as the means ± SE. The differences were considered significant at a p value < 0.05.

3. Results

3.1. GHM sheet impregnated with antagomir-92a increases angiogenesis

There were abundant CD-31-positive endothelial cells in the non-infarct area (posterior LV wall) 14 days after LAD coronary

![Fig. 1. Attachment of gelatin hydrogel microsphere sheet on the area at risk of myocardial infarction in rats. A: gelatin hydrogel microsphere sheet impregnated with antagomir-92a. B; dashed line indicates estimated area at risk of infarction after ligation of the left anterior descending coronary artery. C: gelatin hydrogel microsphere sheet attached on the area at risk of myocardial infarction.](image-url)
artery ligation, but there are virtually no-endothelial cells with positive-immunostaining for BrdU (Fig. 2A). There were only a small number of endothelial cells with positive-immunostaining for BrdU in the infarct border zone 14 days after MI, when the GHM sheet without antagomir-92a was attached on the area at risk of MI. The GHM sheet impregnated with antagomir-92a provoked a robust increase in BrdU-positive endothelial cells in the infarct border zone. Such an increase in BrdU-positive endothelial cells was not observed, when the GHM sheet was impregnated with antagomir-control.

Fig. 2. The effect of the gelatin hydrogel microsphere sheet impregnated with antagomir-92a on angiogenesis. A; the effect of the GHM sheet impregnated with antagomir-92a on endothelial cell proliferation. Red, CD-31; green, bromodeoxyuridine (BrdU); blue, DAPI. GHM, gelatin hydrogel microsphere sheet; Ant-92a, gelatin hydrogel microsphere sheet impregnated with antagomir-92a; Ant-Co, gelatin hydrogel microsphere sheet impregnated with antagomir-control. The arrows indicate BrdU-positive endothelial cells. Bars indicate 20 μm. Lower panel; quantitative analysis for BrdU-positive endothelial cells. Each bar graph indicates mean ± SE of 5 experiments. *p < 0.01 compared to Ant-92a. B; the effect of the GHM sheet impregnated with antagomir-92a on capillary density. Red, CD-31. Bars indicate 20 μm. Lower panel; quantitative analysis for the number of capillaries. Each bar graph indicates mean ± SE of 5 experiments. #p < 0.05 compared to sham, *p < 0.01 compared to Ant-92a.
Capillary density was decreased in the infarct border zone 14 days after LAD coronary artery ligation, when the GHM sheet without antagonim-92a was attached on the area at risk of MI (Fig. 2B). The GHM sheet impregnated with antagonim-92a but not antagonim-control highly significantly (p < 0.01) increased capillary density in the infarct border zone.

3.2 GHM sheet impregnated with antagonim-92a increases cardiac stem cell proliferation

It has been demonstrated that c-kit-positive cardiac stem cells normally reside in the heart and presumably are responsible for replenishing the pool of cardiomyocytes and endothelial cells under normal conditions [18–20]. Therefore, we investigated whether c-kit-positive cardiac stem cells are accumulated in the infarcted tissue. There were no c-kit-positive cells in the non-infarct area (posterior LV wall) 3 days after MI (Fig. 3). There were only a small number of c-kit-positive cells that incorporated BrdU in the infarct border zone 3 days after MI, when the GHM sheet without antagonim-92a was attached on the area at risk of MI. c-kit and BrdU-positive cells were dramatically increased in the infarct border zone 3 days after MI, when the GHM sheet impregnated with antagonim-92a was attached on the area at risk of MI. There was no increase in c-kit and BrdU-positive cells when the GHM sheet impregnated with antagonim-control was attached on the area at risk of MI.

3.3 GHM sheet impregnated with antagonim-92a increases cardiomyogenesis

There was no cardiomyocytes that incorporated BrdU in the non-infarct area 14 days after MI (Fig. 4). There were only a small number of cardiomyocytes that incorporated BrdU in the infarct border zone 14 days after MI, when the GHM sheet without antagonim-92a was attached on the area at risk of MI. BrdU-positive cardiomyocytes were markedly increased by the GHM sheet impregnated with antagonim-92a. These cardiomyocytes were relatively smaller than mature cardiomyocytes as observed in the non-infarct area, and the cluster of BrdU-positive cardiomyocytes formed islands in the infarct border zone. The GHM sheet impregnated with antagonim-control did not increase BrdU-positive cardiomyocytes 14 days after MI, and the island of cardiomyocytes was appreciably smaller than that observed in the GHM sheet impregnated with antagonim-92a.

3.4 GHM sheet impregnated with antagonim-92a reduces infarct size

There was no difference in the area at risk of infarction between the groups 14 days after LAD coronary artery ligation (Fig. 5). When the GHM sheet impregnated with antagonim-92a was attached on the area at risk of MI, the scar area was highly significantly (p < 0.01) reduced 14 days after MI compared to the sheet alone or the sheet impregnated with antagonim-control.

3.5 GHM sheet impregnated with antagonim-92a improves LV function

LV wall motion score index was not different between the groups 1 h after LAD coronary artery ligation (Fig. 6A), suggesting equal degree of LV dysfunction at the time of MI. LV wall motion score index was significantly increased 14 days after LAD coronary artery ligation in all groups. However, deterioration of LV wall motion score index was significantly inhibited, when the GHM sheet impregnated with antagonim-92a but not antagonim-control was attached on the area at risk of MI. LVDd was not different between the groups 1 h after LAD coronary artery ligation (Fig. 6B). LVDd significantly increased 14 days...
Fig. 4. The effect of the gelatin hydrogel microsphere sheet impregnated with antagonim-92a on cardiomyocyte proliferation. Red, α-actinin; green, bromodeoxyuridine (BrdU); blue, DAPI. GHM, gelatin hydrogel microsphere sheet; Ant-92a, gelatin hydrogel microsphere sheet impregnated with antagonim-92a; Ant-Co, gelatin hydrogel microsphere sheet impregnated with antagonim-control. The arrows indicate BrdU-positive cardiomyocytes. Bars indicate 20 μm. Lower panel; quantitative analysis for BrdU-positive cardiomyocytes. Each bar graph indicates mean ± SE of 5 experiments. *p < 0.01 compared to Ant-92a.

Fig. 5. The effect of the gelatin hydrogel microsphere sheet impregnated with antagonim-92a on infarct size. Fibrous tissues (scars) are stained with blue and cardiac muscles are stained with red. GHM, gelatin hydrogel microsphere sheet; Ant-92a, gelatin hydrogel microsphere sheet impregnated with antagonim-92a; Ant-Co, gelatin hydrogel microsphere sheet impregnated with antagonim-control. Lower panel; quantitative analysis for % risk area/left ventricle (LV) and % scar area/LV. Each bar graph indicates mean ± SE of 5 experiments. *p < 0.01 compared to Ant-92a.
after LAD coronary artery ligation in all groups except for the sham surgery group. However, LV dilatation was significantly inhibited, when the GHM sheet impregnated with antagomir-92a but not antagomir-control was attached on the area at risk of MI.

FS significantly decreased 1 h after LAD coronary artery ligation without an intergroup difference (Fig. 6C). FS remained lower in these groups 14 days after the LAD coronary artery ligation compared to the sham surgery group. However, the GHM sheet impregnated with antagomir-92a significantly improved FS compared to the sheet alone or the sheet impregnated with antagomir-control.

4. Discussion

Since miR-92a expression is increased in the ischemic myocardium immediately after MI, and miR-92a inhibits angiogenesis in vitro [12], miR-92a has become a target for cardiac regeneration therapy. The present study demonstrated that attachment of the GHM sheet impregnated with antagomir-92a on the area at risk of MI produced robust angiogenesis in the infarct border associated with cardiac stem cell and cardiomyocyte proliferation. This facilitated cardiac regeneration was associated with reduction of scar formation and improvement of LV function.

We are the first to employ the GHM sheet to deliver antagomir-92a into the heart after MI. The gene therapy system is generally divided into two categories: viral and non-viral vectors. Although viral vectors such as retrovirus, adenovirus and adeno-associated virus are potentially efficient, non-viral vectors have the advantages of less toxicity, less immunogenicity, and easier preparation [21]. So far several methods to deliver genes with non-viral carriers have been developed including naked plasmid DNA injection and complex formation with cationized polymers or cationized liposomes. However, there are several drawbacks with each non-viral vector, including a low efficiency of gene transfection compared with viral vectors. In contrast, cationized gelatin is a very safe molecule in human being, and cationized gelatin microspheres allow controlled release and sustained exposure of RNAs to cells [22]. Furthermore, the present study suggests that the local...
delivery of antagomir-92a using the GHM sheet to the infarcted tissue could reduce the dose of the oligonucleotide up to one-tenth of that administered into systemic circulation as reported by Bonauer et al. [12]. It may minimize potential side effects such as unwanted angiogenesis in off-target organs. To prove the advantage of this technique, exact pharmacokinetics and an optimal dose of antagomir-92a impregnated with the GHM sheet attached on the infarcted heart remains to be investigated.

The present study demonstrated that cardiomyocyte proliferation was enhanced by the GHM sheet impregnated with antagomir-92a. It remains unclear whether proliferation of cardiac stem cells in the infarcted myocardium using the GHM sheet impregnated with antagomir-92a solely depends on enhanced angiogenesis. It is possible that antagomir-92a directly stimulates cardiac stem cells to migrate toward and proliferate in the infarcted myocardium independent of angiogenesis. There have been no previous reports regarding the effects of miR-92a on cardiac stem cell migration and proliferation. However, because angiogenesis is indispensable for cardiomyogenesis [23], antagomir-92a-induced angiogenesis may provide a suitable niche for cardiac stem cell migration, proliferation and differentiation into cardiomyocytes. It should also be noted that cardiac stem cells can differentiate into not only cardiomyocytes but also endothelial cells and vascular smooth muscle cells [18–20]. Therefore, it is speculated that antagomir-92a could promote cardiac regeneration via angiogenesis-dependent and independent mechanisms.

It also remains unknown whether prevention of scar formation is simply due to enhanced cardiomyogenesis or to reduced cardiomyocyte death after MI. The effect of antagomir-92a on cardiomyocyte protection is controversial. Bonauer et al. [12] demonstrated that antagomir-92a had no protective effect on oxidative stress-induced apoptosis in rat neonatal cardiomyocytes, suggesting that cardiomyogenesis is more important in reducing scar formation than preventing cardiomyocyte death after MI. On the other hand, locked nucleic acid-modified antisense miR-92a protected cultured cardiomyocytes from hypoxia/reoxygenation-induced cell death [13]. Besides pro-angiogenic and cytoprotective effects, Hinkel et al. [13] also demonstrated an anti-inflammatory effect of the antisense miR-92a. Thus, it is anticipated that such pleiotropic effects of antagomir-92a synergistically contributes to cardiomyogenesis and culminates in robust regeneration of the heart after MI.

In conclusion, attachment of the GHM sheet impregnated with antagomir-92a facilitates cardiac regeneration after MI in rats. The present study suggests that this technique may be an attractive surgical regimen either alone or in combination with coronary artery bypass graft surgery in patients with acute MI.

**Conflict of interest**

There is no conflict of interest for all authors.

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