Effects of omentopexy combined with granulocyte colony-stimulating factor in a rabbit heart model

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Abstract

Objective: We investigated whether omentopexy combined with subcutaneously administered granulocyte colony-stimulating factor (G-CSF) reduces infarction areas and improves left ventricular dysfunction in a rabbit model of coronary occlusion and reperfusion. Methods: A coronary artery of a male Japanese white rabbit was ligated for 30 min and then reperfused. An omental pedicle graft was fixed onto the myocardial ischemic area after abrading the epicardium. G-CSF (10 μg kg⁻¹ day⁻¹) was subcutaneously administered for 5 days postoperatively. Animals were assigned to groups (n = 7 per group) as follows: group N, saline; group O, omentopexy and saline; group G, G-CSF; and group OG, omentopexy and G-CSF. At 4 weeks postoperatively, left ventricular ejection fraction and left ventricular end-diastolic diameter were evaluated by echocardiography. Harvested left ventricles were stained with Evans blue and triphenyltetrazolium chloride to measure necrotic and fibrotic areas. The arteriolar density in ischemic and nonischemic areas was evaluated. At 7 days postoperatively, the intrathoracic omentum was evaluated (n = 6 per group). Results: Echocardiography at 4 weeks postoperatively revealed significant improvement in the left ventricular dysfunction of group OG. Necrosis and fibrosis in ischemic areas were significantly reduced in groups G and OG. Arteriolar density in the ischemic area and intrathoracic omentum weight were increased largely in group OG than in the other groups. Conclusions: Omentopexy with G-CSF offers more potential benefits for the ischemic heart than G-CSF alone.

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1. Introduction

Treatment for coronary artery disease, including drugs, catheter intervention, and coronary artery bypass grafting (CABG), aims to salvage the myocardium from ischemia by improving blood flow.

The omentum is an organ with rich blood flow that is anatomically easy to graft. This tissue has been applied in thoracic surgery to prevent infections such as postoperative mediastinitis and empyema, and in abdominal surgery to repair and prevent infections due to upper gastrointestinal perforation. However, because angiogenesis from the omentum requires several days [1], it has been considered unsuitable for reperfusion during the acute phase of myocardial ischemia. Nevertheless, in models of acute myocardial infarction (AMI) and chronic ischemic heart disease (IHD), omentopexy using a gelatin hydrogel sheet impregnated with basic fibroblast growth factor [2,3] as well as that after myocardial injections of bone marrow (BM)-derived mononuclear cells [4] have yielded effective results. That is, omentopexy, when combined with stem cells or cytokines with angiogenic effects, can restore blood flow to the ischemic myocardium, rendering such combination therapy potentially useful against IHD.

We therefore examined the effects of omentopexy plus granulocyte colony-stimulating factor (G-CSF), which is currently used to induce neutrophil differentiation in patients with BM suppression. The safety and efficacy of G-CSF have been established. The effects of G-CSF on myocardial ischemia are indirect, and include the differentiation and induction of BM stem cells and of endothelial progenitor cells (EPCs) as well as the promotion of vascular endothelial growth factor (VEGF) release from neutrophils [5]. In addition, G-CSF directly affects the myocardium by accelerating the healing process after AMI due to activation of the matrix metalloproteinase family [6], by exerting antiapoptotic effects mediated by Akt-1 activation during the acute phase [7], and by the Janus kinase-signal transducer and activator of transcription pathway during the chronic phase [8]. Furthermore, the omentum itself produces and secretes more VEGF than other organs; under specific circumstances, such as ischemia and G-CSF administration, VEGF production and secretion are increased.

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and even if the omentum itself is not ischemic, contact with ischemic organs can enhance angiogenesis [9]. When combined with VEGF-2 gene therapy, G-CSF has synergistic effects on the ischemic myocardium [10]. The direct and indirect myocardial protective effects of G-CSF, together with omentopexy, should synergistically enhance omental angiogenic effects, thus conferring further benefits upon the ischemic myocardium.

The present study investigated whether omentopexy combined with G-CSF has synergistically enhanced effects, and whether it can reduce infarct size and improve cardiac dysfunction in an ischemia–reperfusion model.

2. Materials and methods

2.1. Experimental animals and surgical procedures

Ethics and experimental procedures were approved by the Committee for Animal Research and Welfare of Gifu University. Animals received humane care in compliance with Regulations for Animal Experiments in Gifu University.

General anesthesia was induced in male Japanese white rabbits weighing 2.5–2.85 kg (Chubu Kagaku Shizai, Nagoya, Japan) via an intravenous administration of sodium pentobarbital (30–40 mg kg\(^{-1}\)) and additional doses were administered to accomplish the surgical procedures. The animals were intubated and ventilated with room air mixed with a nistered to accomplish the surgical procedures. The animals

2.1.3. Assignation by omentopexy and administration of G-CSF

2.1.4. Sacrifice

After the induction of general anesthesia, 500 IU kg\(^{-1}\) of heparin was administered intravenously and each animal was sacrificed after an overdose of pentobarbital. The wound was reopened to excise the heart, followed by excision of the omentum cut at the diaphragm level in groups O and OG.

2.2. Assessment

2.2.1. Body weight and blood sampling

The animals were weighed and blood samples for peripheral blood cell counts were collected preoperatively, as well as at 7 days and 4 weeks postoperatively.

2.2.2. Time-course assessment with echocardiography

Two individuals, including a certificated physician, performed transthoracic echocardiography (SSD2000, Aloka Co. Ltd., Tokyo, Japan) to preoperatively evaluate left ventricular function [3] at 2 days and at 4 weeks, postoperatively, under general anesthesia. Two-dimensional (2D) parasternal long-axis views of the left ventricle of the middle of the upper-left anterior chest wall were obtained until appropriate images were obtained. Ejection fraction (EF) and left ventricular end-diastolic diameter (LVDD) were measured using the Teichholz method from M-mode images obtained by echocardiography.

2.2.3. Pathological assessment of the left ventricle

Animals were sacrificed at 4 weeks postoperatively. The intrathoracic omentum over the excised heart was peeled off and weighed. The coronary artery was then re-occluded and 1% Evans blue dye was injected from the aorta to determine the area at risk (AAR) under ischemic conditions during coronary artery occlusion [12]. After both atria and the right ventricular free wall were removed, the left ventricle (LV) was sectioned into six slices parallel to the atrioventricular ring, from the ligated portion of the coronary artery to the apex. Four slices, excluding those at each end, were incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C to visualize necrotic areas [11]. After 24 h, all sections were weighed and each slice was photographed to evaluate nonischemic areas stained with blue dye and, among the AAR, white necrotic and unstained non-necrotic areas. The sizes of the AAR and of necrosis were determined as ratios (% of the whole LV and (%) of the AAR, respectively [12]. All specimens were fixed in 10% formalin, embedded in paraffin, and sectioned using a microtome and then, three slices from each animal were stained with hematoxylin and eosin (HE) and Masson’s trichrome (MTC) to evaluate fibrotic areas (% area of LV slice). Five fields for each LV slice were randomly selected in ischemic and nonischemic areas to count arterioles under microscopy (magnification $\times 200$) and thus evaluate angiogenesis. Arterioles were defined as vessels with a diameter of 25–100 $\mu$m.
2.2.4. Pathological assessment of the omentum

To assess the omentum during the early phase after occlusion and reperfusion injury, other animals were assigned to groups O and OG (n = 6 each) and sacrificed at 7 days postoperatively. The heart, with the omentum, was sectioned into three slices parallel to the atrioventricular ring, from the site of coronary arterial ligation to the apex. All slices were fixed in 10% buffered formalin, embedded in paraffin, and sectioned using a microtome. Microscopic sections from groups O and OG were immunohistochemically stained using an anti-human VEGF mouse monoclonal antibody clone (ab28775; Abcam, Cambridge, UK) to examine VEGF expression.

2.3. Statistical analysis

All values are expressed as means ± standard deviation. Data were statistically analyzed using analysis of variance (ANOVA), followed by Bonferroni/Dunn’s post hoc test, respectively. Values of \( p < 0.05 \) were considered statistically significant.

3. Results

3.1. Body weight and peripheral blood cell counts

Body weight did not significantly differ among the four groups preoperatively, or at 7 days, or 4 weeks postoperatively (data not shown).

Peripheral blood cell counts did not significantly differ in any respect among the four groups, except for the white-blood-cell count on postoperative day 7, which was significantly increased in groups G and OG compared with groups N and O (groups N, O, G, and OG, 10.6 ± 1.88, 10.6 ± 1.5, 17.5 ± 4.6, and 20.1 ± 8.4 \( \times 10^{12} \) \( / \)C\(_{6}\) \( / \)C\(_{6}\) \( / \)C\(_{6}\) \( / \)C\(_{6}\), respectively; G vs N, O, \( p < 0.05 \); OG vs N, O, \( p < 0.005 \)).

3.2. Improved LV function in group OG

EF did not significantly differ among the four groups preoperatively or on postoperative day 2, but was significantly more improved in group OG than in groups N, O, and G 4 weeks postoperatively (groups N, O, G, and OG, 41.1 ± 6.1%, 44.4 ± 3.8%, 50.0 ± 3.0%, and OG 56.7 ± 4.1%, respectively; OG vs N and OG vs O, \( p < 0.001 \); OG vs G, \( p = 0.0092 \); G vs N, \( p = 0.001 \); G vs O, \( p = 0.0259 \); Fig. 1(A)).

In addition, LVDD did not significantly differ among the four groups preoperatively and on postoperative day 2, but was significantly more improved in group OG than in groups N, O, and G 4 weeks postoperatively (groups N, O, G, and OG, 13.9 ± 1.5, 12.9 ± 0.8, 12.8 ± 1.9, and 11.2 ± 1.1 mm, respectively; OG vs N, \( p = 0.0012 \); OG vs O, \( p = 0.0268 \); OG vs G, \( p = 0.0357 \); Fig. 1(B)).

3.3. Decrease in necrotic and fibrotic areas in groups G and OG

The AAR (% of whole LV) did not significantly differ among the four groups (groups N, O, G, and OG, 21.7 ± 4.0%, 21.9 ± 3.8%, 20.7 ± 4.7%, and 21.9 ± 8.0%).

4. Necrotic areas at 4 weeks postoperatively in groups G and OG were significantly decreased compared with group N. However, groups G and OG did not significantly differ. Group O tended toward a smaller necrotic area than group N, but the value did not reach significance (groups N, O, G, and OG, 32.1 ± 10.9%, 26.3 ± 9.7%, 20.0 ± 7.0%, and 19.6 ± 8.0%, respectively; G vs N, OG vs N; \( p = 0.02 \), N vs O; \( p = 0.24 \); Fig. 2(A)). Fig. 3(A) shows representative images of TTC and Evans blue dye staining of LV cross-sections.

Fibrotic areas (% area of LV slice) measured by MTC staining 4 weeks postoperatively were significantly decreased in groups G and OG compared with groups N and O. Furthermore, the decrease in the fibrotic areas did not significantly differ between groups G and OG. The fibrotic area tended to be smaller in group O than in group N, but significantly (groups N, O, G, and OG, 19.4 ± 5.5%, 16.1 ± 4.2%, 11.2 ± 3.0%, and OG 11.4 ± 4.1%; OG vs N, \( p < 0.001 \); G, OG vs O, \( p < 0.001 \); N vs O, \( p = 0.051 \); Fig. 2(B)). Fig. 3(B) shows representative images of MTC staining of LV slices.

3.4. Arteriolar density increased the most in group OG

The arteriolar density of the ischemic areas in groups O, G, and OG was significantly increased compared with group N.
(group N vs O, G, and OG, 1.97 ± 0.7 vs 3.06 ± 1.7, 3.01 ± 1.6, and 3.76 ± 2.4 mm²; O and G vs N, p < 0.05; OG vs N, p < 0.001). The increase in arteriolar density was comparable between groups O and G and tended to be the highest in group OG, but not significantly. The arteriolar density did not significantly differ in nonischemic areas among the four groups (groups N, O, G, and OG, 1.71 ± 0.9, 1.90 ± 1.0, 1.74 ± 0.6, and 1.81 ± 0.7 mm², respectively; Fig. 4).

3.5. Assessment of omentum

Immunohistochemical microscopic assessment using anti-VEGF staining at 7 days postoperatively revealed more intensely stained cytoplasm in adipocytes of group OG than in G (Fig. 5(A)).

The intrathoracic omentum weighed 6.2 ± 1.4 and 11.3 ± 1.8 g in groups O and OG, respectively, indicating a significantly greater increment in group OG than in group O 4 weeks postoperatively (O vs OG, p < 0.0001). Preoperative omentum weight (group N, n = 6) was 3.9 ± 1.09 g (N vs O, N.S.; N vs OG, p < 0.0001) (Fig. 5(B)).

4. Discussion

The primary goal of treating coronary artery disease is to improve myocardial blood flow. Conventional therapy, including drugs, catheter intervention and CABG, are all aimed at improving perfusion into ischemic myocardium. However, treatment to regenerate or salvage the necrotic myocardium has yet to be established.

Regenerative medicine such as cell transplantation or protein/cytokine therapy has recently become highlighted for treating IHD. Orlic et al. [13] described that BM stem cells transplanted into the infarcted region of a mouse model of AMI differentiate into myocardial, vascular endothelial, and vascular smooth muscle cells, which enables the use of BM stem cells as a source for myocardial regeneration. With respect to protein/cytokine therapy, G-CSF-induced BM stem cell mobilization in a mouse model of AMI significantly decreased mortality, improved cardiac function, and prevented cardiac remodeling compared with a control group [14]. Cell transplants and protein/cytokine therapy have also been used with omentopexy, and some authors have described preferable effects in AMI and in chronic IHD models [2—4].

We evaluated G-CSF as a therapy combined with omentopexy as it is used to treat post-chemotherapy neutropenia, and it has proven safe and effective. Experimental studies have revealed that G-CSF exerts direct and indirect effects on the ischemic myocardium [5—8]. Furthermore, G-CSF has exerted experimental cardioprotective effects by preventing myocardial ischemic—reperfusion injury after heart preservation in cardiac transplantation [15]. By contrast, Myocardial Regeneration and Angiogenesis in Myocardial Infarction with G-CSF and Intra-Coronary Stem Cell Infusion (MAGIC) cell trials have shown that an intracoronary infusion of G-CSF provokes harmful restenosis after coronary stenting [16,17]. However, we administered G-CSF systemically after performing omentopexy as a new
source of blood supply. We considered that this strategy would avoid the harmful effects of direct intracoronary administration even if coronary restenosis occurred.

We postulated that the effects of this strategy would become evident after 4 weeks as reported by Ueyama et al. [2] and Takaba et al. [3]. Therefore, we evaluated cardiac function and LV histopathology 4 weeks postoperatively. We also assessed VEGF expression by the omentum at 7 days postoperatively according to the peak of VEGF release from neutrophils [5].

The results of our histopathological evaluation of group G (G-CSF alone) was similar to those reported by Minatoguchi et al. [6], and showed a significant decrease in necrotic and fibrotic areas compared with groups N and O. Echocardiography showed that LV dysfunction relatively improved in group G compared with groups N and O. In group OG, the decrease in necrotic and fibrotic areas resembled that in group G, but the arteriolar density of the ischemic myocardium relatively increased compared with groups O and G. In addition, LV dysfunction was the most improved in terms of both parameters compared with the other three groups. In particular, LVd decreased postoperatively only in group OG.

The mechanism of improvement in cardiac function can be explained as follows.

One explanation is the establishment of omental—myocardial blood flow. With omentopexy alone, the establishment of capillary and arteriolar circulation requires 3 and 8 days, respectively [1]. A combination of basic fibroblast growth factor (bFGF) and omentopexy can accelerate this process, and omental—myocardial blood flow thus reduces the infarct area and improves cardiac function [2,3]. Further, significant improvements in the cardiac function of group OG in the present study included reduced infarct size and increased arteriolar density of the ischemic area compared with group O. In addition, anti-VEGF staining of adipocyte cytoplasm in the omentum was better in group OG than in group O on postoperative day 7. These findings suggest that G-CSF accelerates the omental production and secretion of VEGF, establishing earlier omental—myocardial capillary and arteriolar circulations and increasing myocardial blood flow, thus maintaining or improving cardiac function.

Another explanation is improved ischemia and accelerated healing due to the antiapoptotic and angiogenic effects of G-CSF on ischemic myocardial cells. The arteriolar density of the ischemic area increased in groups O and G (Fig. 4), and was further significantly increased in group OG. Moreover, although infarct sizes were similar in groups G and OG, LV function at 4 weeks postoperatively was better in group OG than in group G. We therefore consider that myocardial cell function was better preserved, and blood flow to the ischemic area was improved earlier in group OG than in groups O and G, due to synergistic effects.

The most likely explanation is that the physical and structural properties of omentopexy help to prevent LV dilation. Circumferential LV strain closely correlates with LV dilation as expressed by Laplace’s law: 

$$ T = \frac{P \times R}{M} $$

(where T is the wall tension, P is the pressure difference across the wall, R is the lumen radius, and M is the wall thickness), and is thus inversely proportional to wall thickness [18]. Li et al. reported that angiotensin-converting enzyme inhibitor administration prevents excessive fibrosis and thinning in the post-infarct heart and maintains the structural integrity of the LV wall, thus preventing cardiomegaly [19]. We applied omental serosectomy [20] and epicardial abrasion [1], which resulted in strong graft adhesion 4 weeks postoperatively. That is, infarct size was reduced and myocardial cell function was maintained in group G, but cardiomegaly could not be prevented; whereas in group OG, the omental graft firmly adhered to the myocardium, LV wall thickness was maintained, and LV dilation was structurally prevented.

Further studies are required to confirm these findings and should include angiographic and histological investigations to demonstrate angiogenesis between the omentum and myocardium, further assessment of LV function with a pressure study, further elucidation of the underlying mechanisms (including immunoblot measurements of various cytokines such as VEGF), determination of whether the omentum disturbs LV diastolic movement, and an assessment of contractile function with measurements of dP/dt (max), an indicator of cardiac systolic function, as well as −dP/dt (max), an indicator of cardiac diastolic function. We assessed VEGF expression in the omentum because the omentum itself produces and secretes more VEGF than any other organ [9]. However, we plan to assess VEGF expression in cardiomyocytes in the future.

We could not identify an appropriate chronic ischemic rabbit heart model. Consequently, we adopted an ischemia—reperfusion model and evaluated subacute changes 4 weeks postoperatively and myocardial rescue in the marginal ischemic zone. Hence, an investigation should be undertaken in a model of chronic ischemia.

In conclusion, the LV dilation that inevitably occurs after myocardial ischemia, regardless of rescue with only G-CSF, could be suppressed via the structural and physical support of the omentum that proliferates via G-CSF administration.

References


