A beneficial effect of thymosin beta 4 plasmid on suppression of the extension of myocardial scar formation in rat acute myocardial infarction

Hiroshi KUBO

Division of Thoracic and Cardiovascular Surgery, Department of Surgery, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan

ABSTRACT Thymosin beta 4 (Tb4) is a peptide that exists in various kinds of biotissues. Recently, a report on its restorative and regenerative actions on the myocardium has attracted attention. Several studies have already reported on the myocardial-restorative effect of Tb4 peptide in mice.

Based on the consideration that local injection of a Tb4 plasmid construct might increase the effect of Tb4, in this study, I compared the effect of locally administered Tb4 plasmid with that of Tb4 peptide.

A rat heterotopic abdominal heart transplantation model (Ono-Lindsey method) was employed. At one week after the transplantation, when the transplanted heart in the model rats became stable, both the left anterior descending (LAD) and circumflex (CX) branches of the left coronary artery of the transplanted heart were ligated, which led to the development of a large myocardial infarct. The rats were then divided into four groups; Tb4 plasmid (50 μg/50 μl) group, the Tb4 peptide (4.0 μg/50 μl) group, the control PBS injection group, and the sham group. In each group, injection into the myocardium was given at three or four sites in the myocardial infarct area. The myocardial tissues were harvested 4 weeks later, and the myocardial-restorative effect of Tb4 was assessed. The harvested myocardial specimens were evaluated using Masson Trichrome stain, and the myocardial scar volume rate was examined.

The myocardium scar volume rate was reduced significantly in both the Tb4 plasmid and Tb4 peptide groups: Tb4 plasmid group (21.7% ± 10.5%; n = 8; p = 0.004 vs. control); Tb4 peptide group (22.7% ± 6.9%; n = 7; p = 0.016 vs. control); control group (39.1% ± 13.8%; n = 7; p = 0.020 vs. sham (n = 6).

Significant reduction of the myocardial scar volume rate was observed in both the Tb4 plasmid and Tb4 peptide groups. There was no significant difference between the two groups in terms of the degree of reduction of the myocardial scar volume.

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Key words : **Thymosin beta 4, Cardiac repair, Angiogenesis, Cardiac infarction**

Corresponding author
Hiroshi Kubo
Division of Thoracic and Cardiovascular Surgery, Department of Surgery, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan

Phone : 81 86 462 1111
Fax : 81 86 462 1199
E-mail : hk@med.kawasaki-m.ac.jp

BACKGROUND

Thymosin beta 4 (Tb4) was isolated from the calf thymus approximately 40 years ago. It belongs to a family of highly conserved polar 5-kDa peptides consisting of 40 to 44 amino acids. In most mammalian tissues, Tb4 is the main peptide, representing approximately 70－80% of the total beta-thymosin content[1]. Tb4 is present in very high concentrations in the leucocytes, platelets and macrophages[2]. Beta-thymosins are often referred to as "G-actin sequestering peptides," based on their ability to bind monomeric globular actin (G-actin) in a 1:1 complex and prevent the polymerization of G-actin into actin filaments. In addition to their role as simple actin monomers buffering proteins, recent studies have revealed that beta-thymosins are actually multifunctional peptides involved in cell migration, angiogenesis, wound healing, inflammation, morphogenesis, and tumor metastasis[2,3,12-16]. While investigating the vasculogenic potential of Tb4, Grant and his coworkers[4] found a fivefold increase of Tb4 mRNA expression during morphological differentiation of endothelial cells into capillary-like tubes. Moreover, Malinda *et al* [5] and Grant *et al* [6] reported that Tb4 induces an increase in cell-matrix attachment, proliferation, tube formation, peptide internalization, and rearrangement of the actin cytoskeleton. A recent topic of interest concerning Tb4 is its involvement in the prevention and repair of cardiac damage after myocardial infarction by promoting cardiac cell migration and survival, demonstrated in mice[7]. Other studies have identified Tb4-induced adult epicardial cells as a viable source of vascular progenitors for continued renewal of regressed vessels at a low basal level or for sustained neovascularization following cardiac injury[8]. These findings suggest that Tb4 promotes cardiomyocyte migration, survival and repair, and reduces the extent of acute myocardial damage. Bock-Marquette *et al* [7] reported that Tb4 formed a functional complex with PINCH and integrin-linked kinase (ILK), resulting in activation of the survival kinase Akt (also known as protein kinase B). In mice subjected to coronary artery ligation, Tb4 treatment upregulated the ILK and Akt activities in the heart, enhanced early myocyte survival, and improved cardiac function. Based on the results of these studies, administration of Tb4 for the repair of infarcted myocardium may be more effective and easier than the strategy involving hematopoietic stem cells. Therefore, a rat model of broad acute myocardial infarction was employed to evaluate the regenerative effect of Tb4; a broad acute myocardial infarction was created in a rat heterotopic abdominal heart transplantation model (Ono-Lindsey method), and the myocardial-restorative effect of the Tb4 plasmid and peptide was investigated.

MATERIALS & METHODS

All experimental procedures were performed in accordance with the rules established by the Animal Care and Use Committee of Kawasaki Medical School (06-021, 07-20).

Animal preparation

A total of 56 inbred male Lewis rats (200－250 g, 9－12w) were purchased from Charles River Laboratories, Inc.

Creating a myocardial infarct in a rat heterotopic abdominal heart transplantation model

The rats were anesthetized with sevoflurane (0.5－3%), and a rat heterotopic abdominal heart transplantation model (Ono-Lindsey method)[9] was created.

The vena cavae of the donor hearts were ligated with 3-0 silk sutures. The ascending aorta and main pulmonary artery were transected, leaving 2- to 3-mm long vessel origins attached to the heart. Heparinized Ringer's sodium solution at 4℃ was injected into the coronary arteries bilaterally via

the ascending aorta. The pulmonary vein was ligated after the heart beat completely stopped; the heart was then harvested. The donor hearts were stored in heparinized Ringer's sodium solution at 4℃. Isogenic heterotopic cardiac transplantation was performed by end-to-side anastomoses of the ascending aorta of the heart graft to the abdominal aorta of the recipient. Then, the graft pulmonary artery was anastomosed to the recipient inferior vena cava. The mean cold ischemic time was 46.4 ± 7.45 min. Declamping of the aorta restored the heart beat of the transplanted hearts.

Injection of Tb4

One week after the transplantation, when the transplant heart state in the model rats became stable, ligation of both the LAD and CX branches of the left coronary artery of the donor heart was performed. With these procedures, a broad myocardial infarction was created. The rats were then divided into four groups; a Tb4 plasmid (50 μg/50 μl) group, a Tb4 peptide (4.0 μg/50 μl) group, a control group, and a sham group. In each group, the injection into the myocardium was made into three or four sites of the myocardium of the infarction area. The grafted hearts were harvested four weeks later, and the myocardial scar volume was evaluated.

Measurement of the myocardium scar volume rate

The harvested myocardial specimens were evaluated using Masson Trichrome stain, and the myocardium scar volume was measured. "IPLab for Windows" (Solution Systems), which is an image processing/analysis software, was used. Statistical calculations were performed using a standard t-test for variables with 95% confidence intervals.

RESULT

The control group showed inflammatory cell infiltration in the myocardial scar tissue on HE staining, and the broad myocardial scar formation on Masson Trichrome staining (Fig.3). The scarred myocardium was localized just around the coronary arteries, and the effectiveness of Tb4 peptide was documented (Fig.4). In the Tb4 plasmid group as well as the Tb4 peptide group, the extent of the scar formation was limited (Fig.5). No myocardial infarction occurred in the sham group, because ligature of the coronary arteries was not performed in this group (Fig.6). The myocardial scar volume rate was significantly reduced in the the Tb4 plasmid and peptide groups: Tb4 plasmid (21.7% ± 10.5%; n = 8; p = 0.004 vs. control); Tb4 peptide group (22.7% ± 6.9%; n = 7; p = 0.016 vs. control); control group (39.1% ± 13.8%; n=7; p = 0.020 vs. sham (n = 6) (Figs.7, 8). Significant reduction of the myocardium scar volume was observed in the

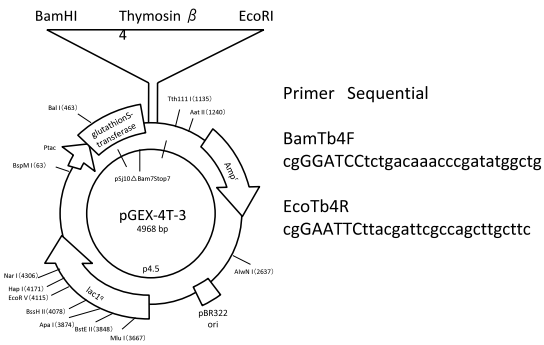

Fig. 1. Structure of plasmid DNA (thymosin beta 4)

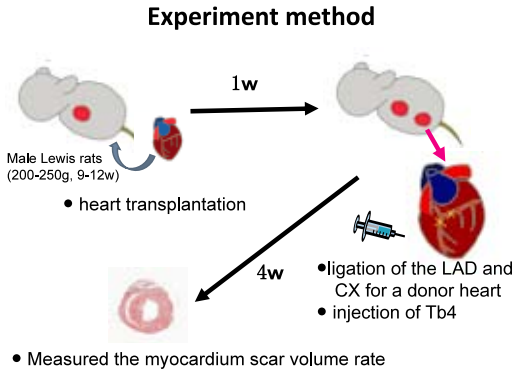

Fig. 2. Flow chart of the experimental method

Fig. 3. Myocardium scar in the control group

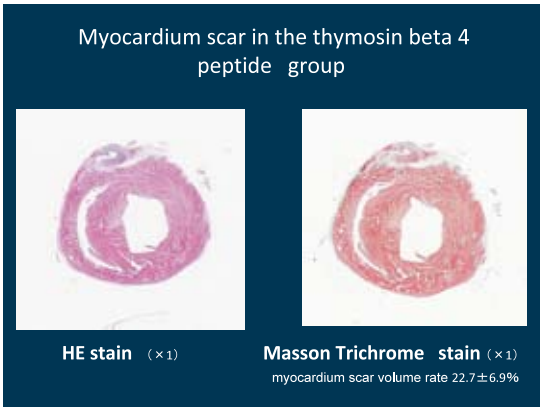

Fig. 4. Myocardium scar in the thymosin beta 4 peptide group

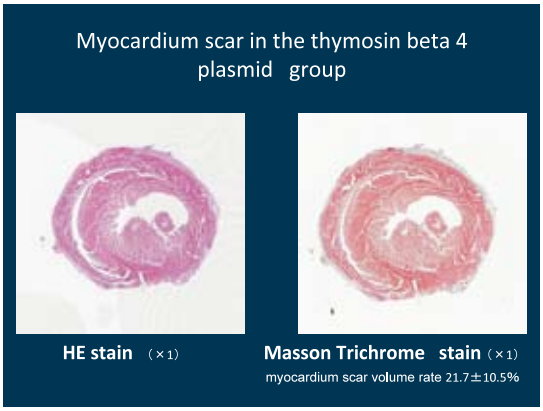

Fig. 5. Myocardium scar in the thymosin beta 4 plasmid group

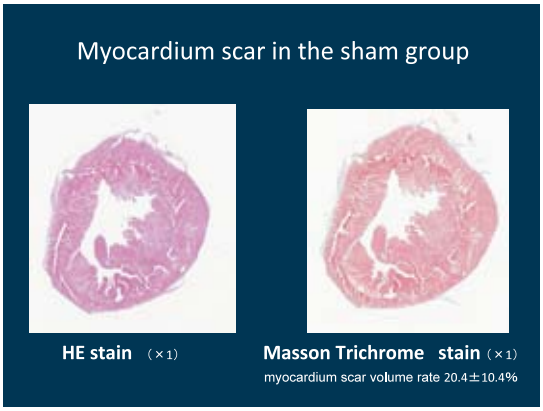

Fig. 6. Myocardium scar in the sham group

plasmid and peptide groups (Fig.8). The rate in the two groups was comparable, with no significant difference.

DISCUSSION

Bock-Marquette *et al* [7] reported that the ability of Tb4 to prevent cell death within 24 h of coronary ligation is probably responsible for the decrease of the scar volume and improved ventricular function observed in mice. Although the activation of ILK by Tb4 is likely to have many cellular effects, activation of Akt may be the dominant mechanism through which Tb4 promotes cell survival. Smart *et al* [8] reported that coronary vasculogenesis is required to maintain cardiomyocyte survival and consequently, appropriate myocardial architecture and cardiac function. The role of Tb4 in coronary vessel development may underlie its reported ability to play a therapeutic role in cardioprotection and repair. Utilizing the role of Tb4 in vascular development in angiogenic therapy for coronary artery disease in the adult heart involves releasing the adult epicardium from a quiescent state and restoring its pluripotency. Various reports have led to Tb4 attracting attention in the field of myocardial regenerative medicine. I considered that use of Tb4 might be more effective and easier than the strategy involving hematopoietic stem cells[10]. I used the rat heterotopic abdominal heart transplantation model to create a large myocardial infarct. The model could be representative of the effect of occlusion of the main trunk of the left coronary artery. I obtained a

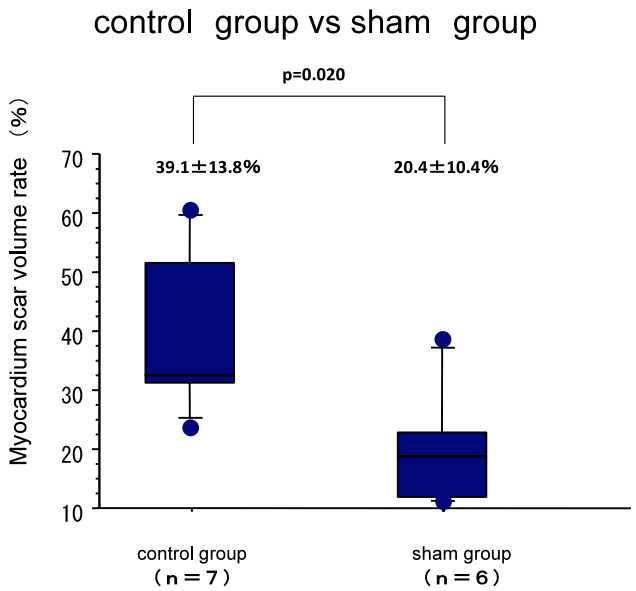

Fig. 7. Myocardium scar volume rate (control group vs sham group)

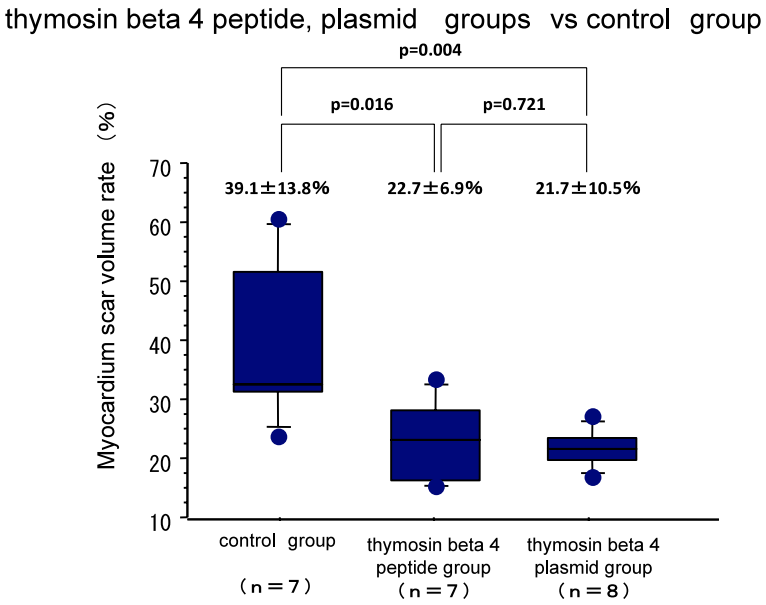

Fig. 8. Myocardium scar volume rate (thymosin beta 4 peptide and plasmid groups vs control group)

good effect in both the Tb4 peptide and Tb4 plasmid groups. I considered that the grafted myocardial tissue might be in a ready state for regeneration, because in this rat heterotopic abdominal heart transplantation model, the transplanted heart works in the condition of non-working beat.

Tb4 can be administered safely to both Sprague—Dawley rats and cynomolgus monkeys at doses of up to 50 mg/kg, and has been shown to be well-tolerated in both species at all dose levels[11]. The dose of Tb4 for this study was fixed based on the report[7] in which 0.4 μg of Tb4 was injected intramyocardially and 150 μg of Tb4 was administered intraperitoneally into mice. The

beneficial effects of Tb4 were documented by the administration of 4.0 μg of Tb4 peptide and 50 μg of Tb4 plasmid in these experiments. On the other hand, some special attention should be paid to the possible induction of malignant neoplasms following Tb4 administration in the clinical setting. Several studies have reported that beta-thymosins perform important roles in modulating the immune responses, vascular biology, and cancer pathogenesis[12,13]. In addition, Cha *et al* reported that Tb4 stimulates tumor metastasis by activating cell migration and vascularization[14]. Tb4 has also been reported to exert anti-inflammatory activity[15,16], therefore, it may also promote tumor growth by inhibiting immune surveillance[14]. Based on the results of these studies, it is necessary to debate the possibility of induction of malignant tumor growth while determining the dose of Tb4 that must be administered in clinical settings. The effect of administration of the Tb4 plasmid on the myocardium has not yet been reported. In this study, the usefulness of Tb4 was evaluated only by measuring its effect on the myocardial scar volume rate. However, the mechanisms, by which Tb4 induces myocardial regeneration, vascularization, ischemic defense, and cell survival promotion during myocardial repair have not yet been clarified. These are important issues that need to be addressed in future studies.

SUMMARY

A large myocardial infarct was created in a rat heterotopic abdominal heart transplantation model (Ono-Lindsey method), and the effects of myocardial administration of Tb4 plasmid and peptide administration in this model were investigated. Significant reduction in the myocardium scar volume rate was observed in both the plasmid and peptide groups. The rates in the 2 groups were comparable, with no significant difference. The results of this study indicate that the use of Tb4 might be more effective and easier than the strategy involving hematopoietic stem cells. Use of Tb4 has begun to attract attention in the field of myocardial regenerative medicine.

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