The effect of thymosin beta 4 plasmid in myocardial infarction

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ABSTRACT OBJECTIVE: Our previous study employing a rat heterotopic abdominal heart transplantation model (Ono-Lindsey method) showed that significantly less myocardial scar tissue was formed in both the Tb4 plasmid and Tb4 peptide groups compared to controls. In the present study, myocardial infarction was induced in the normal working rat heart and the effect of the Tb4 plasmid on myocardial scar formation was examined.

MATERIALS & METHODS: The left anterior descending branches of the left coronary artery of rats were ligated to induce broad antero septal myocardial infarction. Rats were divided into the following 2 groups: Tb4 plasmid (50 μg/50 μl) group and empty vector injection control group. In each group, the drug was injected at 2 or 3 sites in the myocardial infarct area. Myocardial tissue, harvested 4 weeks later, was evaluated using Masson trichrome staining, and the myocardial scar volume was measured. In addition, expression of the plasmid at the local myocardium was detected in the plasmid group 1 week later by immunostaining.

RESULTS: The immunostain revealed Tb4 expression in the myocardial infarct area where the Tb4 plasmid had been injected. Myocardial scar volume was significantly lower in the Tb4 plasmid group (19.5±4.6%, n =21, p =0.019 vs control) than in the control group (23.4±4.7%, n =15).

CONCLUSION: Significantly less myocardial scar tissue was formed in the Tb4 plasmid group compared with the control group. The Tb4 plasmid can reduce myocardial scar volume in myocardial infarction by means of the expression of Tb4.

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

INTORODUCTION

Thymosin beta 4 (Tb4) was first isolated from calf thymus approximately 40 years ago. It belongs to a family of highly conserved polar 5-kDa peptides consisting of 40–44 amino acids. In most mammalian tissue, Tb4 is the main peptide, representing approximately 70–80% of total beta-thymosin content[1]. Tb4 is present in very high concentrations in white blood cells and thrombocytes among other tissues[2]. In addition to

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acting as a simple actin monomer buffering protein, recent studies have revealed that beta thymosins are multifunctional peptides involved in cell migration, angiogenesis, wound healing, inflammation, morphogenesis, and tumor metastasis[2-9]. While investigating the vasculogenic potential of Tb4, Grant and coworkers found that Tb4 mRNA increases 5-fold during the morphological differentiation of endothelial cells into capillary-like tubes[10]. Moreover, both Malinda et al[11] and Grant et al[12] reported that Tb4 stimulates 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 in mice by promoting cardiac cell migration and survival[13]. Other studies have identified Tb4-induced adult epicardial cells as a viable source of vascular progenitors for the continued renewal of regressed vessels at a low basal level or for sustained neovascularization following cardiac injury[14]. These findings suggest that Tb4 promotes cardiomyocyte migration, survival, and repair, and the pathway it regulates may be a new therapeutic target for the treatment of acute myocardial damage. Bock-Marquette et al[13] reported that Tb4 formed a functional complex with particularly interesting new cysteine-histidine-rich (PINCH) proteins 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 ILK and Akt activity in the heart, enhanced early myocyte survival, and improved cardiac function. Based on the results of these studies, the administration of Tb4 for the repair of an infarcted myocardium may be more effective and easier than the conventional strategy involving isolating and implanting hematopoietic stem cells.

In our previous study which used a rat heterotopic abdominal heart transplantation model[9] (the Ono-Lindsey method[15]), significantly less myocardial scar tissue formed in both the Tb4 plasmid and Tb4 peptide groups compared with a control group. In the present study, the effect of the Tb4 plasmid on the suppression of scar formation was evaluated, after myocardial infarction was induced in a normal working rat heart.

MATERIALS AND METHODS

Experimental procedures and protocols were approved by the Animal Research Committee of Kawasaki Medical School, Kurashiki, Japan, and were performed in accordance with institutional guidelines.

Animal preparation

Male Lewis rats (250-300 g; n=36; Charles River Laboratories Japan, Yokohama, Japan) were premedicated with ketamine (90 mg/kg) and xylazine (10 mg/kg) and orally intubated. Respiration was controlled mechanically by Sevoflurane inhalation (1.0-3.0%, with 100% oxygen; Anaesthesia WorkStation, Hallowell EMC, Pittsfield, MA). Body temperature was controlled with an electric heating pad and surgery was performed using aseptic techniques.

Injection of Tb4

After entering the left thoracic cavity by thoracotomy, the left anterior descending branches of the left coronary artery were ligated with 7-0 polypropylene suture (Prolene® ; Ethicon, Inc., Somerville, NJ) and the rats were divided into the following 2 groups: the Tb4 plasmid (50 μg /50 μl) group and the empty vector injection control group. In each group, drugs were injected at 2 or 3 sites of the myocardial infarction area. Myocardial tissue was harvested 4 weeks later, and the suppressive effect of Tb4 on the volume of myocardial scar formed was evaluated. In addition, cardiac tissue from the plasmid group was extracted

1 week after treatment and expression of the plasmid was confirmed by beta galactosidase and immunostaining. Tb4 was obtained from Y. Osawa (Fig. 1, 2).

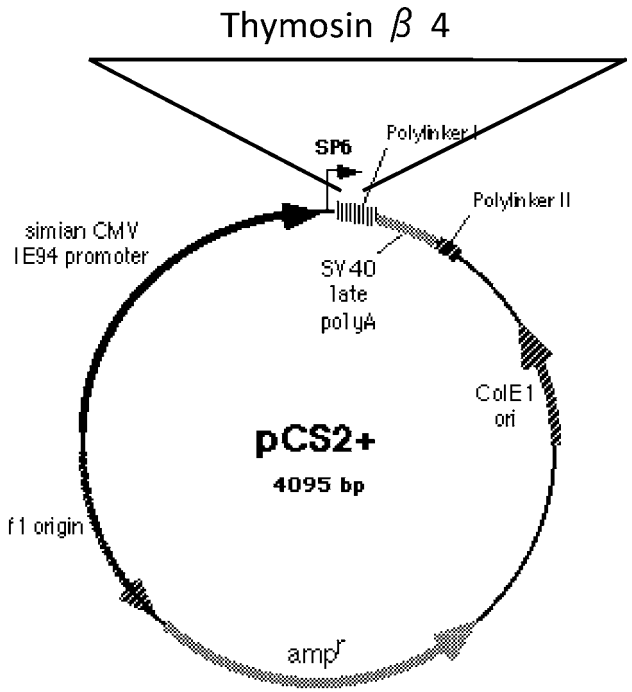

Fig. 1. Structure of the thymosin beta 4 (Tb4) plasmid DNA

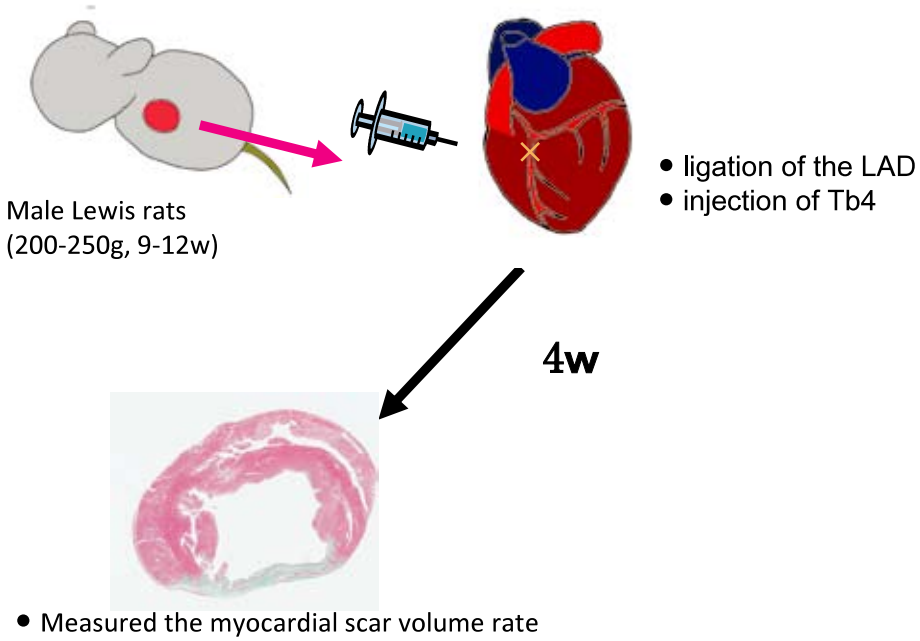

Fig. 2. Experimental method flow chart

Immunohistochemical and morphometric analysis

Myocardial tissue was snap frozen in liquid nitrogen-cooled isopentane and sectioned transversely (10 μm) at the center of the cardiac muscle using a cryostat (Model, Leica Microsystems, City, Country/US state) with an A35 Microtome Blade (Tech-Jam, City, Country/US state) as reported previously[17,18]. Sections were postfixed in 1% formalin in phosphate buffered saline (PBS) for 10 min and immunostained according to the MOM procedure (Vector Mouse on Mouse Kit, Vector Laboratories, City, Country/US state) using rabbit polyclonal antibody (Ab) against caveolin-3 (BD Biosciences, City, Country/US state) using rat FITC-conjugated secondary Ab.

Measurement of volume of myocardial scar formation and statistics

The extracted myocardial specimens were evaluated using Masson trichrome staining, and the myocardial scar volume was measured. IPLab for Windows (Solution Systems, City, Country/US state) software was used for image processing and analysis. Statistical calculations were performed using a standard t-test for variables with 95% confidence intervals.

RESULTS

In beta galactosidase staining of the myocardium, the plasmid was ingested into a cell and stained blue (Fig. 3, arrows). Immunostaining revealed Tb4 expression in the myocardial infarct area, which was strongly stained (Fig. 4, arrows). Tb4 was expressed in the parts of myocardium where Tb4 plasmid was injected, and the expression was observed in one or two cells per one field of vision under the microscope. The volume of myocardial scar tissue was 19.5 ± 4.6% and 23.4 ± 4.7% in the

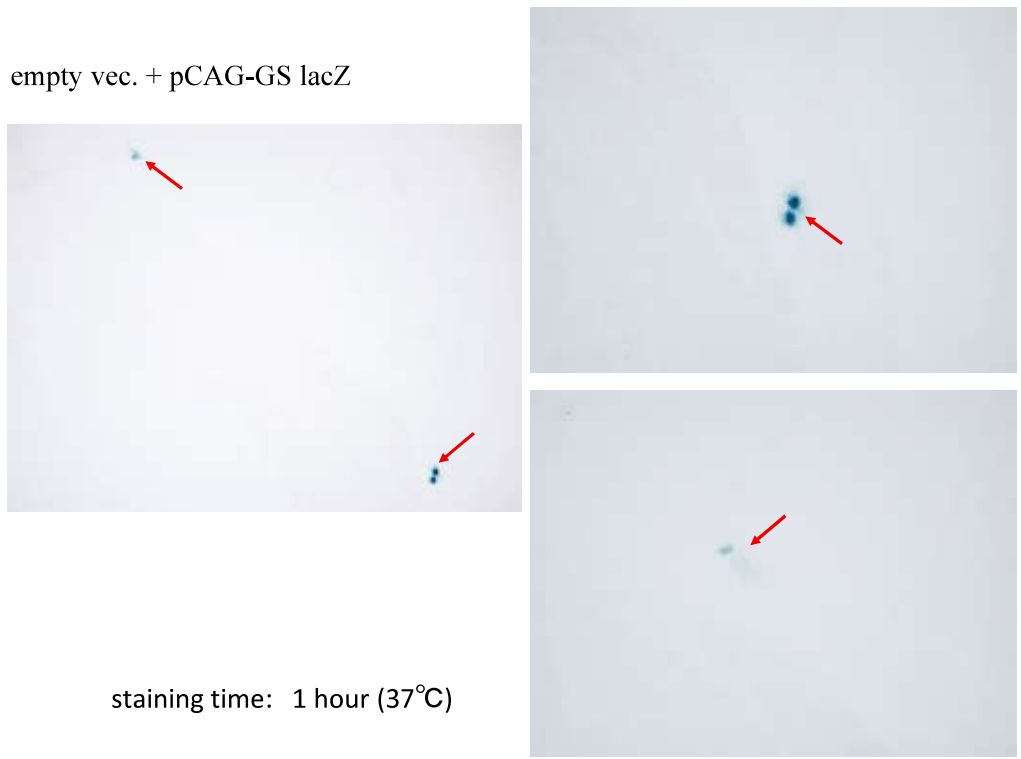

Fig. 3. Beta galactosidase staining of rat cardiac muscle
In beta galactosidase staining of the myocardium, the plasmid was ingested into a cell and stained blue (arrows).

Tb4 plasmid group (n = 21) and the control group (n = 15) respectively. The volume of myocardial scar tissue formed was significantly lower in the Tb4 plasmid group (19.5 ± 4.6%, n = 21, p = 0.019 vs control) than in the control group (23.4 ± 4.7%, n = 15) (Fig. 5).

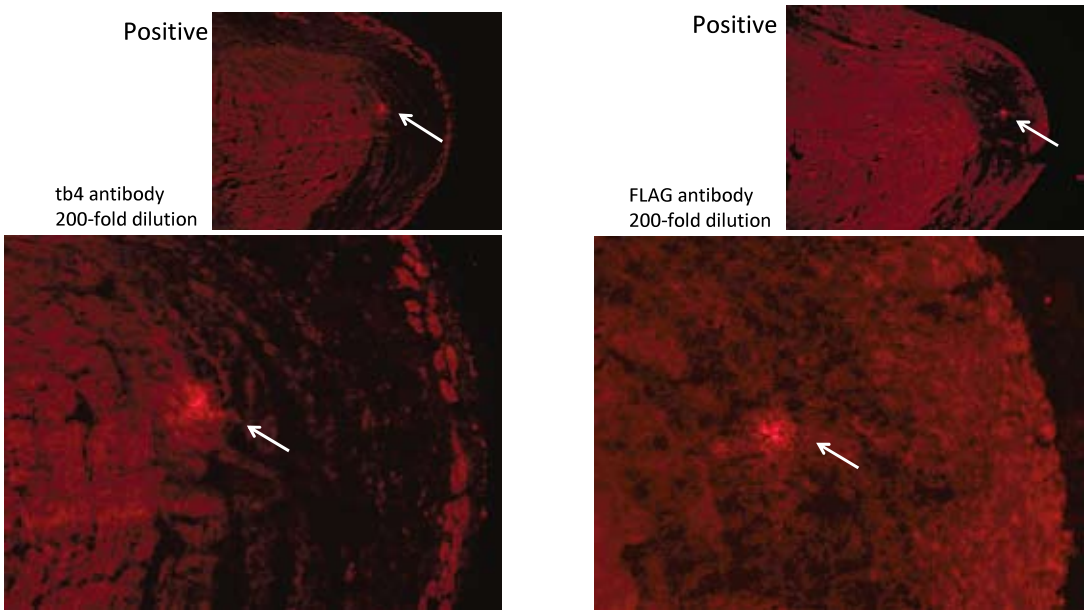

Fig. 4. Immunostaining of rat cardiac muscle
Immunostaining revealed Tb4 expression in the myocardial infarct area, which was strongly stained (arrows). The sites of Tb4 expression corresponded to the parts of the myocardium where Tb4 plasmid had been injected.

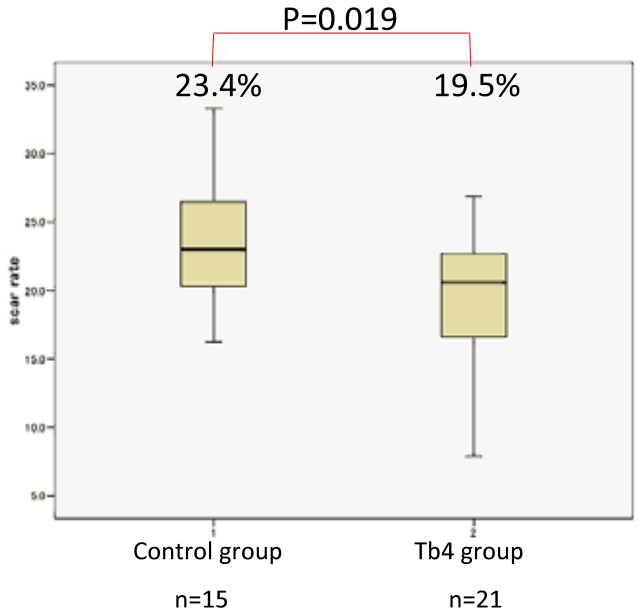

Fig. 5. Comparison of myocardial scar volume between the Tb4 plasmid group and control group

DISCUSSION

Bock-Marquette et al[13] reported that the ability of Tb4 to prevent cell death within 24 h of coronary ligation is probably responsible for the decreased scar volume and improved ventricular function observed in mice. Although Tb4 activation of ILK is likely to have many cellular effects, the activation of Akt may be the dominant mechanism through which Tb4 promotes cell survival. Smart et al[14] 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.

In our previously study, the condition of rats stabilized after a heart transplant in which ligation of both the left anterior descending branches and circumflex branches of the left coronary artery of the donor heart was performed. This model demonstrated the effect of occlusion of the main trunk of the left coronary artery. Consequently, we 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 worked in the condition of non-working beat. In the present study, therefore, myocardial infarction was induced in a normal working heart and the effect of injecting Tb4 plasmid on cardiac repair was examined. As a result, myocardial scar volume was significantly less in the Tb4 plasmid group than in the control group.

Tb4 can be administered safely to both Sprague-Dawley rats and cynomolgus monkeys at doses of up to 50 mg/kg and was well tolerated in both species at all doses tested[16]. The dose of Tb4 for this study was fixed based on report[13] in which 0.4 μg of Tb4 was injected intramyocardially and 150 μg of Tb4 was administered intraperitoneally to mice. In our previous study, acceptable scar volume results were obtained by the administration of 4.0 μg of Tb4 peptide or 50 μg of Tb4 plasmid[16]. Therefore, a similar dose of Tb4 plasmid was used in the present study.

Beta galactosidase and immunostaining revealed the positive effects of introducing Tb4 into the myocardium and visualized Tb4 in the myocardial infarct area. This is the first report documenting the appearance of Tb4 at the same sites where Tb4 plasmid had been injected.

The effect of the Tb4 plasmid on suppression of myocardial scar formation was also demonstrated in this study. However, as only a low quantity of Tb4 was introduced into the myocardium, it is thought that increasing the quantity of Tb4 plasmid is important to induce a greater effect. The mechanism by which Tb4 induces myocardial reformation, vascularization, ischemic defense, and cell survival in myocardial repair remains to be clarified. Such important issues need to be addressed in future studies.

SUMMARY

The effect of the Tb4 plasmid injected into the myocardial tissue on suppression of myocardial scar formation was examined by using a myocardial infarction model in a normal working rat heart. Immunostaining revealed Tb4 expression in the myocardial infarct area where the Tb4 plasmid was injected. As a result, myocardial scar volume was significantly less in the Tb4 plasmid group than in the control group. Therefore, the use of Tb4 is of therapeutic interest in the field of myocardial repair.

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