

# Effects of Tissue-Type Plasminogen Site-Specific Transgene in Gelatin-Coated Dacron on the Fibrinolysis Activity of Rabbit Left Atrium

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**Background:** To investigate the effects of tissue-type plasminogen activator (tPA) gene transfer with left-atrium local positioning on the fibrinolytic activity of rabbit left atrial blood.

**Methods:** A total of 48 rabbits were randomly divided into 3 groups (n = 16): gene therapy, vector control, and blank control groups. Each group was divided into 2 subgroups (8 rabbits in each subgroup) according to the sacrifice time on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days. The tPA mRNA transcriptional level and exogenous tPA protein expression within regional myocardial tissues of the left atrium were detected on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days. After excluding the animals that died, 6 samples of each subgroup were randomly selected for the statistics (n = 6).

**Results:** The tPA activities in rabbit left atrial blood and peripheral blood were also detected. The tPAmRNA and tPA protein expressions within regional myocardial tissues were detected on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days. The tPA activity in left atrial blood in the gene therapy group was higher than the tPA activity of other groups (p < 0.02). No significant differences were observed in the tPA activity of peripheral blood among the 3 groups before surgery. A gelatin-coated Dacron piece, which carried the tPA gene, was implanted in the left atrial appendage.

**Conclusions:** The gelatin-coated Dacron piece could express and secrete tPA proteins in the region, thus enhancing the fibrinolytic activity of left atrial blood.

**Key Words:** Fibrinolytic activity • Gelatin coating • Gene • Left atrium • Tissue-type plasminogen activator

## INTRODUCTION

Patients require anticoagulation therapy after mechanical valve replacement surgery to avoid thrombosis. Current anticoagulation methods require long-term anticoagulant medication and inconvenient administra-

tion, thus causing bleeding and other complications.<sup>1-3</sup> Therefore, transfecting a gene with an anticoagulant function into a target organ by localized gene transfer technology has become a popular approach to enable the target organ to secrete anticoagulant molecules regionally and prevent thrombosis.

The key to gene therapy is the transfer of the exogenous recombination gene into target cells, and successful gene expression in cells requires a suitable vector. The most commonly used vectors are viral and non-viral vectors. Viral vectors include retroviruses, adenoviruses, and herpes viruses,<sup>4,5</sup> which can easily elicit strong immune responses and may be carcinogenic, thus limiting clinical applications. The transfection efficiency of liposome is low in non-viral vectors.<sup>6</sup> Another non-viral vector is the gene activation matrix, which is also called

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the local gene delivery carrier technology.<sup>7</sup> The gene activation matrix combines biological material with plasmid DNA to form a local gene delivery system. Compared with conventional gene therapy, the use of the local gene delivery carrier technology may bypass complex steps in conventional gene transfection, increase the stability of the plasmid in the material, enhance the durability of the target protein, and boost the potency of the therapy.<sup>8</sup>

Therefore, this study applied the local gene delivery carrier technology for the localized transfer of a specific gene with highly efficient anticoagulant factor (tissue-type plasminogen gene) into rabbit left atria. The effect of this method on left atrial thrombus formation was investigated to provide a basis for the genetic anticoagulant therapy of the mechanical valve.

## MATERIALS AND METHODS

### Preparation of gelatin tissue-type plasminogen activator gene coating material

Preparation of gelatin tissue-type plasminogen activator gene coating material was as described elsewhere.<sup>9</sup> First, 5 g gelatin (Sigma-Aldrich, St. Louis, MO, USA) was added to 250 mL 100 mM phosphate buffer solution (PBS) (pH 5.0). The solution was then added to ethylene diamine and ethyl [3-(dimethyl-amino) propyl] carbodiimide hydrochloride (HCL). Thereafter, 5 M HCl was used immediately to adjust the pH to 5.0. The solution was oscillated at 37 °C for 18 h and then dialyzed at 25 °C for 48 h.

The triple-distilled water was removed to prepare the cationic gelatin. First, 50 mg cationic gelatin was added to 50 mL PBS (pH 7.4), mixed with 10 mg plasmid DNA, and gently shaken at 37 °C for 30 min. The above mixture was added to 10% glutaraldehyde cross-linking agent. The mixture was then stored at 4 °C for 24 h to prepare the coating material mixture of the cationic gelatin-DNA. A 0.8 cm × 0.8 cm Dacron slice was placed in the cationic cross-linked gelatin solution containing the pcDNA3.1/tissue-type plasminogen activator (tPA) plasmid for 10 min. The Dacron slice was then air-dried at 20 °C for 15 min. The above procedure was repeated twice followed by air-drying for 20 h. The product was then stored for future use.

Prior to the animal experiments, the Dacron slice was soaked in pcDNA3.1/tPA solution for 3 min and then dried. The above procedure was repeated three times (each Dacron slice contained approximately 800 µg DNA). The same procedure was performed for the vector coating material.

### Animals and grouping

A total of 48 New Zealand big-eared white rabbits with 2.5 kg to 3 kg body weights were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The rabbits were randomly divided into 3 groups (16 rabbits in each group): a gene therapy group (transfected with 800 µg tPA gene), a vector control group (transfected with 800 µg plasmid), and a blank control group (implanted only with the gelatin coating slice without any gene). Each group was divided into 2 subgroups (8 rabbits in each subgroup) on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days.

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

### Modeling and local gene transfection

After body weight was measured, the animal was anesthetized with 2% sodium pentobarbital (35 mg/kg) through the ear vein. The third, fourth, and fifth ribs were then cut along the left edge of the sternum (without damaging the pleura) to expose the pericardial cavity. The bottom of the atrial appendage was clamped, and the top of the atrial appendage was cut open. The prepared Dacron slice was sutured along the inner wall of the atrial appendage. The atrial appendage was then sutured, and hemostasis and chest suture were performed. Thereafter, 200,000 units of penicillin were intraperitoneally injected. After recovery from the anesthesia, the animal was normally fed, and subsequent procedures were performed at different time points. A total of 7 rabbits died during the experiment, and 41 rabbits survived. After excluding the animals that died, 6 samples of each subgroup (n = 6) were randomly se-

lected as the basis for statistical tabulation.

### Specimen collection

Peripheral venous blood was drawn from the ear marginal vein before the application of anesthesia. On the postoperative 3rd and 14th days, the animal was anesthetized and fixed in the supine position, and the pericardial cavity was cut along the original incision. Left atrial blood was extracted, centrifuged, and stored for tPA activity assay. The animal was then sacrificed, and the myocardial tissues around the Dacron slice were immediately extracted and stored in a refrigerator at  $-80^{\circ}\text{C}$  for total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR). For the 2nd subgroup, the myocardial tissues were stored in a refrigerator at  $-80^{\circ}\text{C}$  for protein extraction and immunoblotting (Western blot). For the 3rd subgroup, the myocardial tissues were fixed with 4% paraformaldehyde for pathological examination. For the 4th subgroup, RT-PCR was used to determine the regional myocardial tPA gene transcription level. The transfected myocardial tissues were obtained at each designated time point for RT-PCR. The upstream and downstream primers of the RT-PCR were 5'-CTGGATTCTCTCTGCCGCC-3' and 5'-CCAAAGC TGCTCACGGTGA-3', respectively, with an amplified fragment length of 1000 bp. The upstream and downstream primers of the internal reference  $\beta$ -actin were 5'-TGGATGATGATATCGC CGC-3' and 5'-GTAGATGGGCACAG TGTGGGT-3', respectively, with a product length of 500 bp (Shanghai GeneCore BioTechnologies Co., Ltd, China). The PCR conditions were as follows: pre-denaturation at  $95^{\circ}\text{C}$  for 5 min, denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $54^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. This cycle was repeated 30 times with a final extension at  $72^{\circ}\text{C}$  for 5 min. The amplification products were analyzed by using 1.5% agarose gel. The blank plasmid cloning cells and non-transfected cells were used as the control. The ratio of integrated optical density (A) of the tPA and  $\beta$ -actin of each group was used to calculate the relative content of the target mRNA fragment.

### Western blot detection of exogenous tPA proteins in regional myocardial tissues

The transfected myocardial tissues were obtained at each time point. The Western blotting was performed as described.<sup>10</sup>

### Immunohistochemistry detection of regional myocardial tPA protein expression

The transfected myocardial tissues were obtained at each time point for immunohistochemical staining determination.

### Changes of tPA activity in the left atrium and peripheral venous blood

Blood was obtained from the left atrium and peripheral vein at each time point for the detection of tPA activity by the chromogenic substrate method (Shanghai Sun Corporation, China). The activity of tPA was determined with commercially available chromogenic substrate method kits according to the manufacturer's instructions.

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). For continuous variables, differences among the gene therapy group, vector control group, and blank control group were estimated by ANOVA analysis followed by use of the Bonferroni correction for pairs of means (the significance level was 0.017 after Bonferroni correction). For variables, the differences between 3<sup>rd</sup> and 14<sup>th</sup> days within the same group were estimated by *t* test.

## RESULTS

### Semi-quantitative RT-PCR

On the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days, the electrophoresis of myocardial tissue RT-PCR products revealed that the amplifications of positive bands at 1000 and 500 bp were detected in the gene therapy group. This result was consistent with the expected results. By contrast, only the internal reference, i.e., the 500 bp band, existed in the vector control and blank control groups (Figure 1). The mRNA relative contents on the postoperative 3rd and 14th days were  $0.79 \pm 0.10$  and  $0.52 \pm 0.12$ , respectively. The mRNA relative content of the postoperative 14th day was lower than the mRNA relative content of the postoperative 3rd day but was highly expressed.

### Expression of exogenous tPA

On the 3<sup>rd</sup> and 14<sup>th</sup> days, the expression of exoge-

nous tPA protein could be positively detected in the gene therapy group (tPA molecular weight is 68KD, tPA protein revealed positive bands at 68KD), whereas a negative result was obtained in the control groups (Figure 2).

### Expression of tPA protein with immunohistochemistry staining

On the postoperative 3<sup>rd</sup> day, partial cytoplasm of the myocardial cells were stained brown (Figure 3A). The myocardial hyalomitome in the vector group showed no positive staining on the postoperative 3<sup>rd</sup> day (Figure 3B); Additionally, the myocardial hyalomitome in the control group showed no positive staining on the postoperative 3<sup>rd</sup> day (Figure 3C).

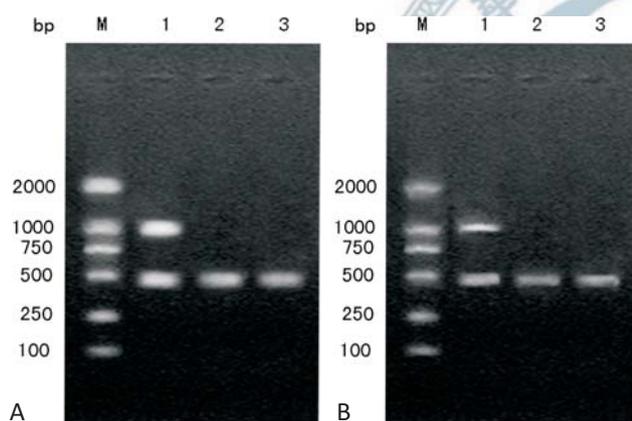
### Changes of tPA activity in the left atrium and peripheral vein

Before operations began, the tPA activities of peripheral blood in the three groups were  $3.61 \pm 0.90$ ,

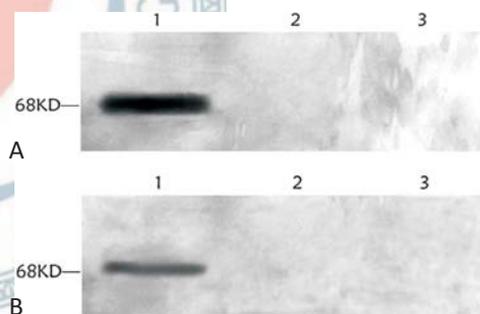
$3.72 \pm 1.12$  and  $3.67 \pm 1.06$  IU/mL, respectively, with no statistically significant differences. The tPA activity of left atrial blood on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days in the gene therapy group was significantly higher than that of the control group. The tPA activity of peripheral blood in the three groups exhibited no significant difference. The tPA activity on the postoperative 3<sup>rd</sup> day was higher than the tPA activity on the postoperative 14<sup>th</sup> day (Table 1).

### DISCUSSION

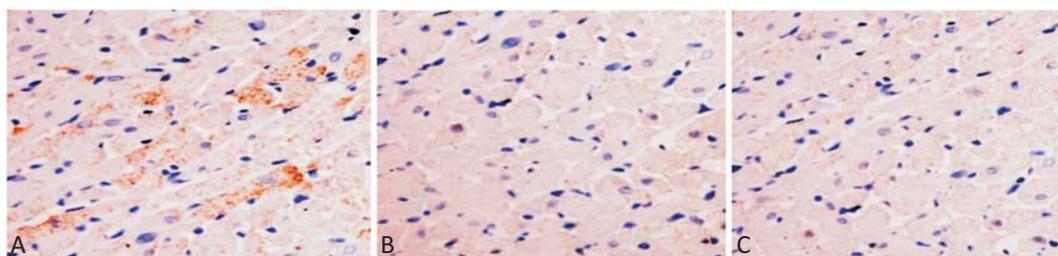
Anticoagulation therapy after mechanical valve replacement has always presented problems to cardiac surgeons. In recent years, reasonable anticoagulation monitoring and other methods have been introduced to reduce anticoagulation intensity and anticoagulation-related complications.<sup>11,12</sup> However, anticoagulation problems still rank the highest in terms of long-term complications. Moreover, lifetime postoperative anticoagulant therapy and monitoring would increase the financial and psychological burden of patients, particu-



**Figure 1.** RT-PCR results of tPA of regional myocardial tissues. (A) On the postoperative 3<sup>rd</sup> day; (B) On the postoperative 14<sup>th</sup> day. M: DNA Marker; 1. gene therapy group; 2. vector control group; 3. blank control group. RT-PCR, reverse transcription polymerase chain reaction; tPA, tissue-type plasminogen activator



**Figure 2.** Western blot results of tPA of regional myocardial tissues. (A) On the postoperative 3<sup>rd</sup> day; (B) On the postoperative 14<sup>th</sup> day. 1. gene therapy group; 2. vector control group; 3. blank control group.



**Figure 3.** tPA protein expression with IHC staining. (A) Partial myocardial hyalomitome was stained brown on the postoperative 3<sup>rd</sup> day ( $\times 400$ ); (B) The myocardial hyalomitome in vector group showed no positive staining on the postoperative 3<sup>rd</sup> day ( $\times 400$ ); (C) The myocardial hyalomitome in the control group showed no positive staining on the postoperative 3<sup>rd</sup> day ( $\times 400$ ). tPA, tissue-type plasminogen activator.

**Table 1.** The postoperative changes of tPA activity in the 3 groups ( $\bar{x} \pm s$ , n = 6)

Group	tPA activity in left atrial blood (IU/ml)	tPA activity in peripheral blood (IU/ml)
Gene therapy group		
3 <sup>rd</sup> day	8.36 ± 1.85* <sup>#</sup>	3.75 ± 1.05
14 <sup>th</sup> day	5.93 ± 1.51*	3.69 ± 0.89
Vector control group		
3 <sup>rd</sup> day	3.74 ± 1.03	3.69 ± 0.92
14 <sup>th</sup> day	3.81 ± 0.96	3.75 ± 1.13
Blank control group		
3 <sup>rd</sup> day	3.69 ± 0.87	3.70 ± 1.12
14 <sup>th</sup> day	3.79 ± 1.12	3.72 ± 1.08

Note: \*  $p < 0.017$ , GenTher vs. VectCon, GenTher vs. BlanCon; <sup>#</sup>  $p < 0.05$ , 3<sup>rd</sup> vs. 14<sup>th</sup> after operation. tPA, tissue-type plasminogen activator.

larly in the vast rural and remote areas of China. Given the lack of appropriate medical conditions, postoperative anticoagulation could not be effectively monitored and treated, thus resulting in surgical failure. Therefore, developing a mechanical valve without the need for oral anticoagulants would be an important priority.

The development of molecular biology techniques and constant cloning technology of target genes has provided alternatives to solve anticoagulation problems by gene therapy. The thrombolytic agents of genetic engineering have been used clinically,<sup>13,14</sup> with tPA exhibiting a specific thrombolytic effect that can potentially treat the main pathological changes caused by thrombosis. However, this gene engineering tPA has a short half-life in blood, and the effective therapeutic dose is up to 1 mg/kg to 2 mg/kg. Moreover, thrombotic diseases generally require long-term medication, thus resulting in high costs. Systemic medication can also cause bleeding easily. Topical application is complex and short-acting, thus leading to limited tPA application. Therefore, tPA gene therapy has received considerable attention.

Eton et al.<sup>15</sup> implanted tPA gene-transplanted vascular smooth muscle cells onto an artificial stent surface and then implanted this stent in animals. The tPA gene expression continued for seven months and significantly improved the thrombotic capability of the graft. Studies have shown that the first three months after artificial mechanical valve replacement surgery is the peak pe-

riod for the occurrence of thromboembolism because the body is in a hypercoagulable state during this period. By contrast, the endothelialization process of the sewing ring of the prosthetic valve would take several weeks, thus facilitating the deposition of platelets on the annulus with sutures. Therefore, we assumed that coating the artificial mechanical valve annulus (Dacron ring) with the tPA gene could prevent thrombosis via the regional sustained secretion of tPA.

To investigate the transfection feasibility of tPA gene-carrying coating material onto regional myocardial cells, this study implanted the tPA gene-carrying gelatin-coated Dacron slice into the left atria of rabbits. The results showed the local expression of tPA mRNA in the left atrial myocardium and the expression of exogenous tPA protein on the postoperative 3<sup>rd</sup> and 14<sup>th</sup> days. This result was not observed in the control groups. After the localized transfection of the tPA gene, the tPA activity of the left atrial blood increased compared with the control groups, whereas tPA activity in the peripheral blood did not change significantly. This result indicated that the proposed method was effective for local gene transfer. The coating method successfully transferred tPA genes to the left atrium, thus maintaining the continuous regional expression and secretion of the active tPA protein and preventing thrombosis without affecting the tPA activity in the peripheral blood.

Common methods of transferring exogenous genes into the heart and blood vessels include direct myocardial injection and site-specific transgene in the coronary arterial cavity. However, these methods are inefficient, have short durations, and exhibit limitations. The development of gene vector research has enabled the combination of biomaterials and plasmid DNA to form a local gene delivery system with a type of plasmid DNA-carrying biological material called the regional gene delivery vector.<sup>16,17</sup> Gelatin is a natural polymer material that can effectively serve as a local gene delivery carrier. Experimental results showed that gelatin was a pharmaceutical carrier with the characteristics of biodegradability, bioabsorbability, non-toxicity, and low immunogenicity.<sup>18,19</sup> Researchers have associated gene gelatin to metal brackets and successfully mediated gene transfer onto the vessel wall.<sup>20</sup> However, this approach was dependent on the adsorption capability of a gelatin in

carrying a gene. Thus, transfection efficiency was low and could not achieve a controlled release. In this experiment, tPA protein expression within the regional myocardial tissues of the left atria of rabbits was still observed on the postoperative 14th day because we combined the gene with the gelatin coating material to realize a continuous release as the material degrades. This approach avoided the drawbacks of the above methods. In this method, DNA was wrapped with cationized gelatin, thus forming positively charged nanoparticles that can easily be used by cells.<sup>9,21,22</sup> Since the pcDNA3.1/tPA itself is negatively charged, it would be expected to be complex with the cationized gelatin. If the pcDNA3.1/tPA cationized gelatin complex has a positive charge, the charge will promote the internalization of the pcDNA3.1/tPA into cells because the cell surface charge is negative. Probably, such a complex formation would facilitate the transfection of pcDNA3.1/tPA, resulting in gene expression. The mechanism of gene expression by the present release system of plasmid DNA should be clarified by further investigation. In the present experiment, we observed that the tPA activity in left atrial blood on the postoperative 3<sup>rd</sup> day was higher than that on the postoperative 14<sup>th</sup> day. Additionally, we also observed that the tPAmRNA relative contents in the tissue samples on the postoperative 3<sup>rd</sup> day were higher than on the postoperative 14<sup>th</sup> day. The change between the tissue samples and the blood samples was identical. We hypothesized that the reason tPA activity decreased on the postoperative 14<sup>th</sup> day was the reduction of tPA gene release from gelatin tissue-type plasminogen activator gene coating material.

In this study, the duration of gene expression was not observed in the long-term. Only a preliminary study was performed on the transfection feasibility of a Dacron slice coated with tPA gene-carrying gelatin onto regional myocardial cells. This study served as the preparatory work for the next development and in vivo animal experiments on the use of gelatin-coated tPA cDNA gene valves.

## CONCLUSIONS

In conclusion, the present data demonstrate that the gelatin-coated Dacron piece could express and se-

crete tPA proteins in the region, thus enhancing the fibrinolytic activity of left atrial blood. The effect of this method on left atrial thrombus formation was investigated to provide a basis for the genetic anticoagulant therapy of the mechanical valve.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest related to the publication of this manuscript.

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