Biomimetic Elastin Fiber Patch in Rat Aorta Angioplasty

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ABSTRACT: Introduction: Vascular grafts significantly contribute to advances in vascular surgery, but none of the currently available prosthetic grafts have elastin fibers similar to native arteries. We hypothesized that a novel elastin patch could be produced after a rat decellularized thoracic aorta elastin fiber scaffold is implanted subcutaneously in rats; we tested this novel elastin patch in a rat aortic arterioplasty model. Methods: Sprague–Dawley rats (200 g) were used. Rat thoracic aortae were decellularized and sectioned at a thickness of 30 μm. A single elastin fiber scaffold was fabricated as a net (5 × 5 mm²), and then a three-layer scaffold was constructed to make a new patch. The hyaluronic acid–sodium alginate (HA/SA) hydrogel was fabricated by reacting sodium SA, HA, and CaCO₃, and then the hydrogel was added to the patch to secure the elastin fibers. The patches were implanted subcutaneously in rats and harvested at day 14. The elastin patches were then implanted into the same rat’s aorta and harvested at day 14; a decellularized rat thoracic aorta (TA) patch was used as a control. Sections of the retrieved patches were stained by immunohistochemistry and immunofluorescence. Results: The elastin fibers could be secured by the hydrogel. After 14 days, the subcutaneously implanted elastin patch was incorporated into the rat tissue, and H&E staining showed that new tissue had formed around the elastin patch with almost no hydrogel left. After implantation into the rat aorta and then retrieval on day 14, H&E staining showed that there was neointima and adventitia formation in both the TA and elastin patch groups. Both patches showed a similar histological structure after implantation, and immunofluorescence showed that there were CD34- and nestin-positive cells in the neointima. In both groups, the endothelial cells expressed the arterial identity markers Ephrin-B2 and dll-4; almost one-third of the cells in the neointima were PCNA-positive with rare cleaved caspase-3-positive cells. Conclusion: We demonstrated a novel approach to making elastin fiber scaffold hydrogel patches (elastin patches) and tested them in a rat aorta arterioplasty model. This patch showed a similar healing process as the decellularized TA patch; it also showed potential applications in large animals and may be a substitute for prosthetic grafts in vascular surgery.

INTRODUCTION

Several different kinds of grafts are used in vascular surgery, such as prosthetic Dacron, expanded poly(tetrafluoroethylene) (ePTFE), biological decellularized materials such as bovine or porcine pericardial, acellular vascular grafts, and autologous vein grafts. Novel biomaterials have also been developed and have shown exciting results in clinical trials. Although these grafts have significantly contributed to the advances in vascular surgery, none of these grafts have elastin fibers such as are found in native arteries, and most grafts are not autologous.

Making better vascular grafts is the dream of both vascular surgeons and scientists. To make an autologous vascular graft, the idea of using the foreign body response to biomaterials within the animal’s own body to produce a graft is promising and practical. Autologous vascular grafts made from the cellular remodeling of fibrotic tissue after an object is implanted subcutaneously have shown excellent results in animal models and in two human patients. The Nakayama group published several studies about using the subcutaneous embedding method to produce autologous grafts with good performance. Recently, the same group presented the initial 3-year results of the first-in-human study of internal shunt restoration using completely autologous vascular grafts. Although stenosis occurred after 3–4 months and percutaneous transluminal angioplasty was required, this novel concept of an internal shunt was a pioneer in vascular surgery. This concept can greatly contribute to the advancement of vascular surgery. However, these grafts could not mimic the structure of arteries because there were no elastin fibers in the graft.

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Elastin fibers are the most important part of arteries. We previously showed that decellularized rat thoracic aorta (TA) contains preserved elastin fibers.15 A hyaluronic acid−sodium alginate (HA/SA) hydrogel scaffold is biocompatible and can be broken down in the body. We have shown that hydrogels can be used as a drug delivery system. 16 Therefore, we sectioned decellularized rat TA to generate elastin fibers. Then, an elastin fiber scaffold was fabricated from the elastin fibers and secured by the hydrogel. A novel elastin patch was made by implanting this elastin fiber scaffold subcutaneously in rats. We tested this novel elastin patch and compared it with a decellularized TA patch in a rat aortic arterioplasty model.

RESULTS

The procedure of making an elastin patch was shown in illustration photographs (Figure 1). The hydrogel was transparent (Figure 2A). After the decellularized rat thoracic aorta was sectioned, the elastin fibers were white; after the hydrogel was added into the elastin fiber scaffold, the elastin fibers were secured (Figure 2A). Then, the elastin fiber scaffold with the hydrogel was implanted subcutaneously in the rat abdomen. Immediately after implantation, the elastin fibers can be seen clearly; after 14 days, the patch was incorporated into the rat tissue, and almost no hydrogel remained (Figure 2B). The decellularized TA patch was implanted as a control.

The elastin patches were harvested carefully and compared with decellularized rat thoracic aorta (TA) patches. H&E staining showed new tissue formed on both sides of the TA patch and there were rare cells infiltrated into the patch. In the elastin patch, the cells infiltrated into the elastin fiber interspace with almost no hydrogel left (Figure 2C). Verhoef’s Van Gieson (EVG) staining showed a similar pattern, and elastin fibers could be seen in the H&E and EVG staining.
cells can infiltrate into the elastin fibers; since the elastin fiber could not degenerate in the animal body, so there was no loss of elastin fibers. There was a similar area of elastin fibers in the TA patch and the elastin patch (Figure 2D). We then measured the suture retention test of the native TA patch, the decellularized TA patch, and the elastin patch; although there was smaller suture retention of the elastin patch compared to the native and decellularized TA patch (Figure 2E), it was still strong enough to withstand the rat aortic pressure after implantation (Figure 2F).

The elastin patch was then implanted into the rat aorta, and after 14 days, the elastin patch was incorporated into the rat aorta with no dilation and no thrombosis formation (Figure 2F). H&E staining showed that there was neointima and adventitia formation in both the TA and elastin patch groups, and elastin fibers could be seen in the elastin patch groups with cell infiltration; the only difference was that the elastin fibers in the elastin patch groups were disorganized (Figure 3).

Then, we examined the neointimal cell composition. Endothelial cell progenitors participate in the process of neointimal formation after patch implantation, and immunohistochemistry showed there were CD34- and nestin-positive cells in the neointima. Most of the CD34- and nestin-positive cells were on the surface of the neointima; some cells were CD34 and nestin dual positive and there was no difference between the patches in terms of CD34-positive cell number (Figure 4A,B). Immunohistochemistry showed α-actin-positive cells in the neointima, and there was no difference in the number of α-actin-positive cells between the TA and elastin patch groups (Figure 4A,B). Immunofluorescence showed...
there was a line of vWF-positive cells on the luminal side, and α-actin-positive cells can be seen below the vWF-positive cells (Figure 4A,B). There were also a few CD68-positive cells in the neointima in the elastin fiber patch group (Figure 4A). We then examined the neointimal endothelial cell identity and found that they acquired arterial and venous identity after patch angioplasty. In both groups, the endothelial cells expressed the arterial identity markers Ephrin-B2 and dll-4, and there was no difference in Ephrin-B2- and dll-4-positive cells between the TA and elastin patch groups (Figure 5A,B). Neointimal cell proliferation contributed to the thickness of the neointima; in both groups, almost one-third of the cells in the neointima were PCNA-positive, with no significant difference (Figure 6A,B); there were a few cleaved caspase-3-positive cells in the neointima in both groups (Figure 6A).

**DISCUSSION**

In this research, we showed the feasibility of constructing a biomimetic elastin fiber patch for use in a rat arterioplasty model. This biomimetic elastin patch showed a similar healing process to the decellularized thoracic aorta patch.

In addition to the conventional vascular grafts used, various studies have been carried out on vascular grafts made by subcutaneous implantation. Qiu et al. found that the formation of fibrotic conduits around subcutaneously implanted mandrels involved fibroblasts and transdifferentiation of inflammatory cells. They decellularized and coated the fibrotic conduits and achieved a high patent rate. Endothelial progenitor migration was observed during endothelialization, and the authors demonstrated successful application of a fibrotic matrix for regenerative engineering. Dahl et al. tested grafts in a baboon arteriovenous access model and a dog peripheral and coronary artery bypass model and showed that tissue-engineered vascular grafts could be used to provide readily available synthetic grafts. Syedain et al. engineered an arteriovenous graft and observed no calcifications, loss of burst strength, outflow stenosis, or overt immune response. They also tested the performance of completely biological, decellularized engineered allografts in a sheep model. The grafts were implanted interpositionally in the femoral artery of sheep, and the authors showed the potential application of these “off-the-shelf” grafts. Furukoshi et al. used caged molds to prepare more robust biotubes, and the authors implanted the biotubes into beagle femoral arteries. Perfect patency was obtained with little stenosis and no aneurysmal dilation. However, none of these grafts had elastin fibers, which are an important component of arteries.

We used decellularized elastin fibers (30 μm) from decellularized rat thoracic aortae and fabricated them into three-layer patches. After 14 days of subcutaneous implantation, this novel elastin patch had a structure similar to the native artery and showed a similar healing process as the decellularized TA patch. Since elastin fiber scaffolds can be made in any size or shape and implanted subcutaneously, this concept of using elastin fiber scaffolds can make patches of different sizes as needed. The hydrogel completely degenerated
after 14 days of implantation in the rat body, which could be seen clearly since the hydrogel can be easily recognized by hematoxylin and eosin staining. 16

This method has many merits. First, it can be used to make grafts of different sizes, different shapes, and different thicknesses. Second, this method does not produce a decellularized patch; it has been recellularized after subcutaneous implantation, and thus it will have decreased immunoreaction and inflammation after reimplantation in an artery or vein. Third, in the future, we plan to test the application and effectiveness of this method for making tube grafts of different sizes to prevent diameter mismatches. 16

We previously showed a similar patch healing process of different prosthetic and biological materials, such as bovine pericardial patches, 22,23 polyester patches, 17 decellularized human saphenous vein patches, 18 decellularized rat thoracic aorta patches, 24,15 and patches from plants. 25 They showed similar healing processes, such as cell infiltration into the patch, endothelial cell infiltration, cell proliferation, and neointima and adventitia formation. This elastin patch also showed a similar patch healing process. In this research, we used the hydrogel to secure the elastin fiber scaffolds. Because the elastin fibers were fabricated piece by piece, the hydrogel could effectively secure the elastin fiber during subcutaneous implantation; this hydrogel can be safely used in animals and can also be used as a drug delivery system to accelerate cell migration and infiltration into the elastin, similar to the native extracellular matrix. 16,25

There are also some limitations of this research. First, we only tested patch angioplasty, and future experiments to show the application of tubes are needed. Second, we only used small animals, and large animals should also be tested. Third, longer observation times after patch placement are essential. Third, since this process needs a period of subcutaneous implantation, it needs preparation when compared to other vascular grafts. Fourth, the mechanical property is still lower when compared to decellularized TA; future modification to enhance the mechanical properties should be tested. Fifth, this is only a preliminary animal experiment, there is still a very long way to apply this method in human clinical trials.

■ CONCLUSIONS

In summary, we demonstrated a novel approach to making elastin fiber scaffold hydrogel patches (elastin patches) in a rat aorta arterioplasty model. This graft has potential applications in large animals and may be a substitute for prosthetic grafts in vascular surgery.

■ METHODS

This study was approved by the First Affiliated Hospital of Zhengzhou University Animal Care and Use Committee. All animal care complied with the Guide for the Care and Use of Laboratory Animals. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed.

Rat Thoracic Aorta (TA) Decellularization. Rats were anesthetized with 10% chloral hydrate (intraperitoneal injection), the rat chest was opened, the TA was collected, and the surrounding fat and tissues outside the adventitia were dissected away. Decellularization was performed as previously described; briefly, the TA was incubated in 250 mL of CHAPS buffer (8 mM CHAPS, 1 M NaCl, and 25 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS)) for 12 h, washed for 60 min, and then incubated in 10 mL of sodium dodecyl sulfate buffer (1.8 mM sodium dodecyl sulfate, 1 M NaCl, and 25 mM EDTA in PBS) for 24 h, followed by washing with PBS to completely remove the detergent. 26,27

Elastin Fiber Scaffold Patch (Elastin Patch) Fabrication. The hyaluronic acid—sodium alginate (HA/SA) hydrogel was fabricated by reacting sodium SA, HA, and CaCO3 as previously described. Briefly, SA (3.2 × 105 to 2.5 × 105 Da2, Sigma-Aldrich) was dissolved in Dulbecco’s modified Eagle’s medium (DMEM)/F12 culture medium (HyClone, HyClone Laboratories) to yield a 0.5% solution; HA (4000 Da, Sigma-Aldrich) was distributed in the SA solution, and then the CaCO3 suspension was introduced into the HA/SA solution, followed by a gluconic acid/lactone solution (GDL; Sigma-Aldrich). After adding the GDL, the cross-linking reaction was triggered, and HA/SA hydrogels were obtained after a 0.5 h reaction.

Decellularized TA was embedded in OCT and sectioned (20 μm). Then, elastin fibers were fabricated around a 5 × 5 mm2 single layer patch, and three single layers were piled up to make a thicker three-layer patch. The hydrogel was added into the interspace of the patch to secure the elastin fibers. The patches were implanted subcutaneously in rats and then harvested at day 14 (Figure 1). Ten elastin patches were fixed and sectioned for histology. Ten elastin patches were implanted into rat aortas, and the patches were retrieved 14 days later for analysis. As a control, decellularized TA patches were also implanted subcutaneously and harvested at day 14, and the TA patches and elastin patches were compared by histology.

Suture Retention Test. Suture retention of the native TA, decellularized TA, and elastin patch was measured as previously described. 28 Briefly, suture retention testing was performed on rectangular specimens (5 cm × 3 cm) clamped at the edges and located opposite to an 8-0 Prolene suture anchored 5 mm from the edge; the suture loop was pulled until the suture tore through the patch, and the tension was recorded.

Rat Aorta Patch Arterioplasty Model. A rat aortic arterioplasty model was used as previously described. 22 Briefly, the rat aorta was exposed, and a 3 mm arteriotomy was made. Patches (4 mm × 2 mm) were sewn to the aorta using running 11-0 nylon sutures; the patches included decellularized thoracic artery (TA) patches and elastin patches. Then, the clamps were removed, and the abdomen was closed. 17,22 The decellularized TA patch was used as a control. The patches were harvested for analysis as described below.

Tissue Analysis. The rats were anesthetized, and tissues were fixed by transcardial perfusion of PBS followed by 10% formalin. The samples were fixed, removed, and then embedded in paraffin and sectioned (4 μm thickness). The tissue sections were deparaffinized and stained with hematoxylin and eosin (H&E, Baso, Zuhuai, China) according to the manufacturer’s recommendations.

Immunohistochemistry. The sections were heated in citric acid buffer (pH 6.0, Beyotime, Shanghai, China) at 100 °C for 10 min for antigen retrieval and then treated with 0.3% hydrogen peroxide for 30 min. The sections were incubated overnight at 4 °C with primary antibodies (Table 1). After overnight incubation, the sections were incubated with the appropriate secondary antibodies (Table 1) for 1 h at room temperature and then treated with a 3,3′-diaminobenzidine

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Positive cell numbers were counted and blindly reviewed independently by three professional pathologists.

**Immunofluorescence Analysis.** The sections were incubated overnight at 4 °C with primary antibodies (Table 1) diluted in dilution buffer (Beyotime, Shanghai, China). The sections were incubated with secondary antibodies (Table 1) for 1 h at room temperature, after which the sections were stained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI, Solarbio, Beijing, China) to mark the cellular nuclei. Positive cell numbers were counted and blindly reviewed independently by three professional pathologists.

**Statistical Analysis.** The data were expressed as the mean ± SEM. Statistical significance was determined by one-way ANOVA, Tukey’s multiple comparison test, or t-test. P-values less than 0.05 were considered significant. The data were analyzed using Prism 6.0 software (GraphPad Software; La Jolla, CA).

### Table 1. Antibodies Used in the Experiment

| Primary Antibody | vendor | lot number | concentration |
|------------------|--------|------------|---------------|
| α-actin          | Abcam  | ab5694     | IF/IHC: 1:100 |
| CD68             | Abcam  | ab31360    | IF/IHC: 1:100 |
| PCNA             | Abcam  | ab29       | IF/IHC: 1:100 |
| cleaved caspase-3| cell signaling | 9,661 | IHC: 1:100 |
| dll-4            | Abclone| A12943     | IHC: 1:100    |
| Ephrin-B2        | Abclone| A12961     | IHC: 1:100    |
| CD34             | Abcam  | ab81289    | IF:1:100      |
| nestin           | Abcam  | ab 11306   | IF:1:100      |
| CD90             | Abcam  | ab225      | IF:1:100      |
| vWF              | Abcam  | ab11713    | IF:1:100      |
| Secondary Antibody |        |            |               |
| HRP goat anti-rabbit | Beyotime | A0208 | 1:100         |
| HRP goat anti-mouse | Beyotime | A0216 | 1:100         |
| 488 goat anti-mouse | AbClonal | A0703 | 1:200         |
| CY3 goat anti-rabbit | AbClonal | A0077 | 1:200         |
| 488 donkey anti-rabbit | AbClonal | A0355 | 1:200         |
| rhodamine donkey anti-λgoat | AbClonal | A0069 | 1:200         |

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