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Treatment of rabbit carotid aneurysms by hybrid stents (microporous thin polyurethane-covered stents): Preservation of side-branches

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Abstract

Objective: We sought to determine the patency of normal arterial branches from the covered segments of an artery after stenting.

Background: Most intracranial aneurysms occur at arterial branching points (bifurcations, side-branches, or perforators). The post-stenting patency of normal arterial branches from the covered segments of the artery is important. We have previously developed a hybrid stent with micropores to prevent early parent artery occlusion by more early endothelialization, and mid- to long-term parent artery stenosis by control of intimal hyperplasia after aneurysm occlusion.

Methods: We created aneurysms in 10 rabbits by distal ligation and intraluminal incubation of elastase within an endovascularly trapped proximal segment of the common carotid artery. All animals were treated with hybrid stents having micropores. Four animals were observed for one month and three each for three and 12 months. The patency of the side-branches of the subclavian artery was evaluated angiographically and in some cases, histologically.

Results: Aneurysms were completely occluded at all time points other than 12 months. The subclavian artery and brachiocephalic artery were patent, without significant stenosis. All the side-branches of the subclavian artery detected on the preoperative angiogram remained patent at the final assessment.

Conclusion: The use of hybrid stents for aneurysm repair and side-branch patency seems to be effective, as per the long-term results obtained in an animal model.

Keywords

Aneurysm, intravascular stent, polyurethane, porosity, endothelium, intimal hyperplasia

Introduction

Most intracranial aneurysms occur at arterial branching points (bifurcations, side-branches, or perforators). Simple covered stents are associated with the highest rate of complete aneurysm occlusion. However, their use in the human intracranial circulation is limited by access issues and the possibility of occluding small perforating end-arteries. It is important to assess the patency of normal arterial branches arising from the covered segments of the artery after implantation of simple covered stents. We established rabbit carotid aneurysms by intraluminal incubation of elastase within an endovascularly trapped segment of the proximal common carotid artery (CCA),[1,2] and we implanted hybrid stents equipped with micropores in these aneurysms.[3] Besides studying the occlusive effect of the hybrid stents in the aneurysms, we examined the patency

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of the side-branches arising from the right subclavian artery (SA), both angiographically and histologically.

Materials and methods

All experiments were performed on Japanese white rabbits (3.5–4.2 kg) and conducted in accordance with the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research, Chicago, IL) and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86–23, revised 1985; National Institute of Health, Bethesda, MD). The research protocol (no. 12010) was approved by the ethics committee of the National Cerebral and Cardiovascular Center Research Institute.

The aneurysms were first created and then subjected to stent implantation two to four weeks later under general anesthesia, which was induced by the intramuscular injection of 0.2 ml/kg ketamine chloride (10%) and 0.3 ml/kg xylazine, and additional dosage was determined on the basis of the animal's movements.

Fabrication of hybrid stents (Figure 1)

A stainless mold (diameter, 1 mm) was dipped in a tetrahydrofuran solution and allowed to dry. Subsequently, a balloon-expandable coronary-artery bare stent (Momo stent: diameter, 3 mm; length, 20 mm; Japan Stent Technology, Okayama, Japan) was mounted on the cover film of the mold, and again, it was dipped in the solution and dried. The thickness of the cover film was restricted to approximately 30 μm. Micropores were then made in the cover film using a KrF excimer laser apparatus (L4500; Hamamatsu Photonics, Shizuoka, Japan). Micropores are circular, with a pore diameter and interpore distance of 100 μm and 250 μm, respectively, such that they achieve an opening ratio of 23.6% (calculated) after full expansion.[3] The uncoated stent was a bare stent with an opening ratio of 86%. The outer surface of the film of the microporous stent grafts was coated with argatroban (500 $\mu g/cm^2$), which was applied using a methanol solution (1% w/v); the solvent was subsequently volatilized for physical adsorption. The fabricated hybrid stent was then remounted on the delivery balloon system (Thrill Slalom PTA dilatation balloon catheter; diameter, 3 mm; length, 2 cm; Cordis, J & J, Europa, N. V., Netherlands).

Preparation of aneurysms in rabbits (Figure 2(a))

Aneurysms were constructed in 10 female rabbits by employing a previously described method,[1,2] with a few simple modifications. The right CCA was surgically isolated, ligated distally, and controlled proximally with 2.0 silk sutures before a 5-Fr detachable sheath introducer (Medikit, Tokyo, Japan) was induced and passed retrograde to the midportion of the CCA (approximately 3 cm cephalad to the origin of the CCA). A hemostatic valve of the sheath (Medikit, Tokyo, Japan) was detached, and a rotational and hemostatic triple connecter was connected to the sheath placed in the CCA. Through the sheath, an occlusive microballoon (Naviballoon; Kaneka, Tokyo, Japan) (Titan percutaneous transluminal coronal angioplasty (PTCA) dilatation balloon catheter: diameter, 4.0 mm; length, 9 mm; Cordis, J & J, Miami, Florida, USA) and subsequently a microcatheter (Excelsior SL10; Striker, Tokyo, Japan) were advanced near the CCA origin (Figure 2(b)). Porcine elastase (Sigma, Tokyo, Japan) was mixed with nonionic contrast medium to obtain a 25% dilution. Elastase (6.8 units/mg/0.25 cc) was injected and incubated in the isolated CCA for 20 min, while the balloon was inflated at the origin of the right CCA. Next, the microcatheter and deflated microballoon were removed. Angiography was performed to confirm the dilation of the affected CCA. The CCA was ligated with

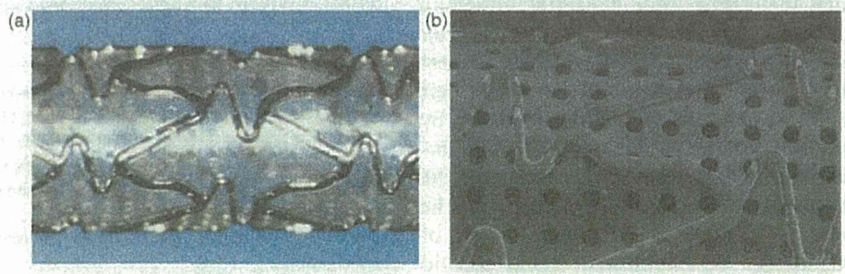

Figure 1. A hybrid stent is shown. Micropores (with a pore diameter and interpore distance of 100 μm and 250 μm, respectively, to accomplish an opening ratio of 23.6% after full expansion) are placed in a polyurethane membrane of approximately 30-μm thickness on a bare coronary stent (a coronary Momo stent, Japan Stent Technology). (a) Macroscopic image and (b) scanning electron microscopic image.

a 2.0 silk thread after sheath removal, and the skin was closed by discontinuous knots with a similar thread.

Endovascular technique

No drugs, such as antiplatelets, were administered during the study. Endovascular treatment was performed via the right femoral artery, at two to four weeks after the creation of the aneurysm. The femoral artery was exposed and ligated distally with a 2.0 silk thread. After pharmacological dilation of the artery with 8 mg of papaverine chloride, a 19-G puncture needle was used to introduce the 0.032″ mandrel; then, a 4-Fr 10-cm sheath (Clinical Supply, Tokyo, Japan) was advanced into the right femoral artery under fluoroscopic guidance.

The 4-Fr catheter was navigated into the brachiocephalic artery (BCA) with a 0.035″ guidewire. Digital subtraction angiography (DSA) showed the BCA, SA, and aneurysm. A balloon catheter (Thrill Slalom PTA dilatation balloon catheter; diameter, 3 mm; length, 2 cm; Cordis, J & J, Europa, N.V., Netherlands) crimped with our hybrid stent was passed through the sheath and navigated into the BCA. Using fluoroscopy and road mapping, we advanced the hybrid stent over the balloon catheter across the aneurysmal neck and inflated the balloon. Post-procedural DSA was performed. The balloon catheter was removed, leaving the hybrid stent in place. Subsequently, the sheath introducer was carefully and slowly pulled out. The femoral artery was ligated with two sutures of a 2.0 silk thread previously placed around the artery, and the skin was closed discontinuously with a similar thread.

Final angiography and harvesting

After the observation period, angiography was performed using a 4-Fr sheath placed at the left CCA in all animals. The left CCA was exposed and ligated distally. A 19-G puncture needle was used to introduce the 0.032″ mandrel; then, a 4-Fr, 10-cm long sheath (Clinical Supply, Tokyo, Japan) was advanced to the left proximal CCA under fluoroscopic guidance. A DSA series was performed to evaluate the final angiographic result. Occlusion of the aneurysms and patency of preoperatively detected side-branches of the artery was evaluated angiographically. Thereafter, the animals were subjected to euthanasia by

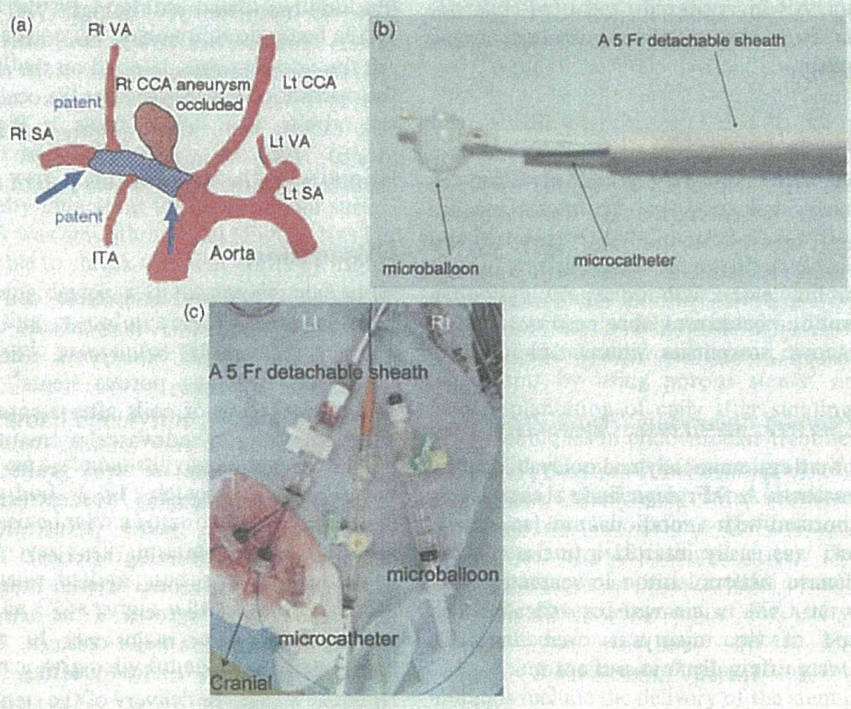

Figure 2. A schema of the aneurysm occlusion in the rabbit using a hybrid stent (arrows) implanted through the femoral artery. Branches such as the vertebral artery, internal thoracic artery, and costocervical artery are indicated (a). Approach to the right CCA via a rotational and hemostatic triple connector. A microcatheter and microballoon are inserted from each hemostatic valve (b). Entrapment system in the right CCA. A microballoon and microcatheter through a 5-Fr detachable sheath (c). CCA: common carotid artery; VA: vertebral artery; ITA: internal thoracic artery; SA: subclavian artery.

intravenous injection of potassium chloride. The BCA, the right SA, vertebral artery (VA), some other arterial branches, and the treated aneurysm were resected en bloc and placed directly in a 4% paraformaldehyde phosphate-buffered saline solution for rapid fixation. Some of the samples were evaluated histologically to determine the patency of the branches.

Histology

Histological examination was performed in some cases, by using a cross-sectional sample (hematoxylin and eosin (H&E) stain) obtained from the stented portion of the aneurysm to confirm the patency of the side-branches.

Results

A balloon catheter crimped with our hybrid stent was easily and smoothly passed through the sheath, navigated into the BCA and right SA via the aorta, and inflated at the neck of the aneurysm. The hybrid stent covered and occluded the aneurysm instantly or after a few minutes. All the side-branches of the right SA observed preoperatively appeared patent (without any significant stenosis) on the angiograms obtained at one, six, and 12 months.

Stent graft and its remounting

The stent struts were completely embedded within the cover film, thereby indicating that the luminal surface of the stent graft was smooth and flat (Figure 1(a) and (b)). It was possible to shrink the stent grafts by using a hand-held crimping device, without any damage to the cover film. Stenting procedures were performed using standard endoscopic procedures without difficulty.[3,4]

Fabrication of carotid aneurysms (Figure 2(c))

All aneurysms were systematically and easily created by our modified method. A 5-Fr detachable sheath introducer, later connected with a rotational and hemostatic triple connector, was easily inserted into the affected CCA. An occlusive balloon and microcatheter were navigated into the CCA origin without difficulty. The size and shape of the aneurysms were variable, although they were originally funnel-shaped.

Occlusion of the aneurysm (Table 1)

At 12 months, all aneurysms but one were completely occluded, with four aneurysms being occluded at one month; three, at three months (Figure 3(a) and (b)); and two, at 12 months (Figure 4(a) and (b)). At the 12-month follow-up, one aneurysm remained patent because of distal movement of the hybrid stent itself from the aneurysm neck.

Table 1. Results.

		Aneurysm	Branches
Months	Numbers	Occlusion	Patency
1	4	4	All[a]
3	3	3	All[a]
12	3	2[b]	All[a]

All aneurysms but one were occluded at 12 months: four aneurysms at one month; three at three months; and two at 12 months. At 12 months, one aneurysm was patent because of distal movement of the stent graft itself from the aneurysm neck at the initial stent placement. Side-branches were the right vertebral artery, internal thoracic artery, costocervical artery, and other small branches. All side-branches drawn preoperatively on the angiogram were visible after occlusion and before the rabbits were euthanized.

[a]Vertebral artery, internal thoracic artery, costocervical artery, etc.

[b]Open in one, due to stent migration.

Preservation of side-branches

The side-branches were the right VA, internal thoracic artery, costocervical artery, and other small branches. All the side-branches detected on the angiogram before the operation were visible after the occlusion and before the rabbits were killed (Table 1; Figures 3(a,b) and 4(a,b)). H&E staining revealed that the orifice of the branch from the right SA was patent (Figure 4(c)).

Discussion

Although Guglielmi detachable coil systems (GDC coils) have been widely accepted and used in the treatment of intracranial aneurysms, primary stenting of aneurysms by using porous stents[5] or stent grafts[6] and implantation of coils after stenting[7–10] are emerging techniques in endovascular treatment.

The development of stent grafts for intracranial aneurysms is challenging. Most intracranial aneurysms occur at branch points (bifurcation, small side-branches, and perforating arteries). The maintenance of the patency of normal arterial branches originating from the covered segment of the artery after stenting across the lesion is a major concern. The salient issues influencing the use of stent grafting for aneurysms in humans include the delivery of the stent graft, degree of cover porosity, configuration of the aneurysm, curvature of the parent vessel, and presence or absence of perforating end-arteries.[2]

A simple bare stent has no efficacious occlusive effect on aneurysms, although it raises a few concerns about

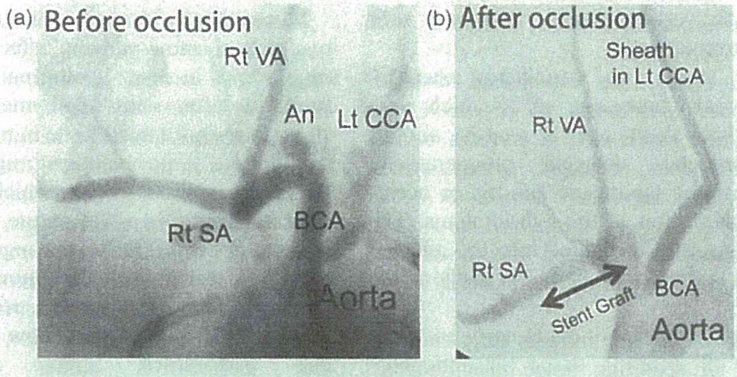

Figure 3. At three months after occlusion, the aneurysm was still occluded (before (a) and after (b)). Side-branches, such as the vertebral artery and costocervical artery, were patent. An arrow shows a stent graft placed a cross the aneurysmal neck (b). CCA: common carotid artery; VA: vertebral artery; BCA: brachiocephalic artery; SA: subclavian artery.

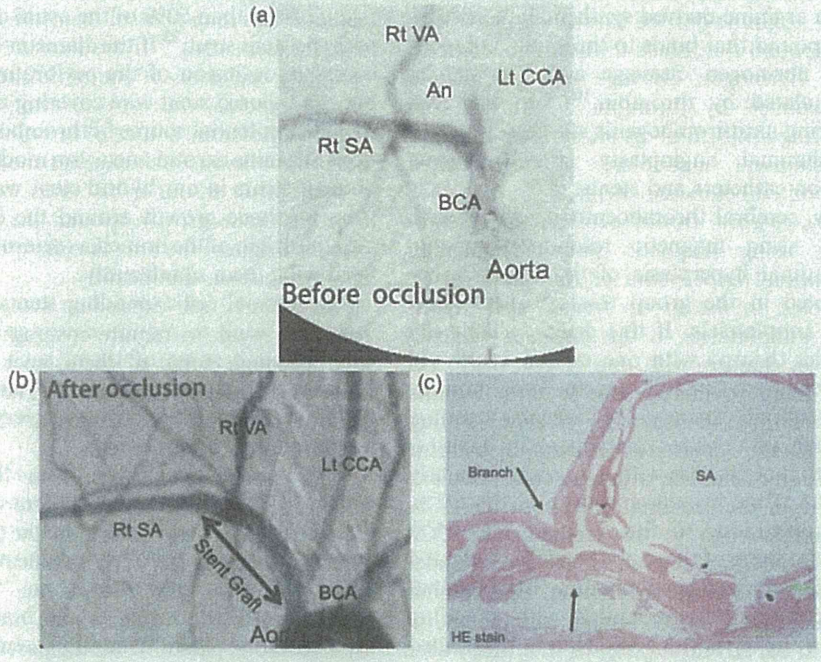

Figure 4. At 12 months after occlusion, the aneurysm was completely occluded (pre (a) and post (b)), while the vertebral artery, internal thoracic artery, and costocervical artery remained patent. An arrow shows a stent graft placed across the aneurysmal neck (b). A cross-sectional specimen (H&E stain) showed that the origin of the branch (arrows) from the subclavian artery was patent at 12 months (c). CCA: common carotid artery; VA: vertebral artery; BCA: brachiocephalic artery; SA: subclavian artery; H&E stain: hematoxylin and eosin stain.

the occlusion of side-branches. In contrast, a simple covered stent has sufficient occlusive effect on the aneurysms, but it can occlude the side-branches. To compensate for the disadvantages of simple covered stents, we have been developing a hybrid stent. This hybrid stent consists of the commercially available balloon-expandable coronary-artery stent with a thin SPU film (by dip-coating method) on which micropores have been created by the excimer laser ablation technique, followed by coating with argatroban.[4] We have developed a hybrid stent with micropores to prevent early parent artery occlusion by more early endothelialization and administration of argatroban, and mid- to long-term parent artery stenosis by control of intimal

hyperplasia after aneurysm occlusion, compared with those in a simple covered stent.

In the current study, we established elastase-induced rabbit carotid aneurysms, all of which were occluded by our hybrid stents until 12 months, and all the small side-branches detected preoperatively remained patent without significant stenosis or occlusion even after implantation of the hybrid stents. The hybrid stent was smoothly navigated into the affected BCA and SA from the femoral artery, as easily as the bare stents.

Our microporous membrane induces early endothelialization capable of secreting tissue plasminogen[11] within one week of stent graft implantation,[3] thereby avoiding early thrombosis and controlling mid- to long-term hyperplasia.[12] Polyurethane has excellent elastomeric properties and is clinically used as a material for manufacturing blood pumps and arterial grafts; it also exhibits no toxicity or little biodegradation.[13–15] Argatroban, an arginine-derived synthetic low-molecular-weight compound that binds to thrombin, competitively inhibits fibrinogen cleavage and the platelet activation stimulated by thrombin.[16,17] It has been used for preparing antithrombogenic surfaces in percutaneous transluminal angioplasty (PTA) devices, including balloon catheters and stents.[18,19]

In this study, cerebral thromboembolic events were not evaluated using magnetic resonance imaging (MRI). Less intimal hyperplasia of the parent artery was focally noted in the group treated with hybrid stents without antiplatelets. If this device is clinically used, antiplatelet therapy with one or two drugs will be necessary for approximately one to three months, as per the conventional therapy after vascular stenting.

The aneurysms were created experimentally by intraluminal incubation of elastase within an endovascularly trapped segment of the proximal portion of the CCA, with slight modifications to the method. The CCA branches from the BCA of the rabbit, and it was ligated beyond the point of elastase incubation. Endoluminal digestion of the internal elastic lamina, with spreading of elastase up to the adventitia, results in a thin-walled aneurysm, as observed in humans.[20] Aneurysm formation occurred over a two-week period, after which the animals were treated with the respective therapies. The rabbit aneurysm model with elastase has some characteristic features: (1) long-term patency in untreated animals, (2) simulating aneurysm morphologies that place a high shear stress on the aneurysmal neck, (3) similar size in aneurysm diameter and parent artery diameter, (4) maintenance of the integrity of the endothelium of the aneurysmal cavity, and (5) short construction time and easy reproduction.[21] The rabbit aneurysm is histologically similar to the human saccular aneurysm and was useful for the current study.

Mechanisms of branch occlusion by stents include the longitudinal snow-plowing effect, stent jailing (early stage), and in-stent neointimal growth (late stage) during or after stent deployment.[22] Cerebral vessels with aneurysms tended to be minimally atherosclerotic. Radial force in the stent strut might be less than that in the intracranial PTA stent, which reduced the longitudinal redistribution of the plaque. Our hybrid stent had a porosity appropriate for the regulation of the in-stent neointima growth even at 12 months (personal communication). The arteriovenous pressure gradient is the driving force of the blood flow through the branches and perforators under normal conditions. Additionally, long-term remodeling of the artery and its lumen, in response to the presence of the intraluminal prosthesis, is less likely to result in complete occlusion of the jailed branch.[22] Experimental evidence in dogs showed that vessels (small muscular branches from the VA) comparable to human perforators tend to remain patent if less than 50% of the ostial diameter is covered with the stent strut.[23] If the diameter of struts is 100 μm, complete occlusion of the perforators may not occur, but the exposed stent wire covering the perforators will remain a potential source of thromboembolic complications in canine carotid aneurysm models.[24] The diameter of stent struts in our hybrid stent was less than 50 μm. Fibrotic tissue growth around the filaments extended into the origin of the branches (external arteries) without narrowing them significantly.

Intracranial self-expanding stents generate a radial force too weak to require coverage with cover materials, although some of them, such as Neuroform or Enterprise, are recommended. Therefore, we selected balloon-expandable coronary-artery stents for the preparation of hybrid stents.

The branch originating from the aneurysm sac, which is treated with a flow diverter (pipeline embolization device), is kept patent in the presence of a flow demand through it.[25] The significant shrinkage of the aneurysm gives the branch an "infundibular-like" appearance at its origin, or the branch is represented by a tortuous takeoff from the parent artery in place of the initial aneurysm. A slow occlusion process for the treatment of ruptured aneurysms is yet to be developed.

The stent developed by us is a hybrid of a bare stent and a simple covered stent; this hybrid nature helps regulate the porosity and enables early intraluminal endothelialization and late inhibition of the intimal hyperplasia. Experimentally fabricated canine carotid aneurysms were occluded with the placement of our hybrid, self-expanding, stent grafts at one,[4] six, and 12 months (personal communication). A porosity of 23.6% was found to be suitable in the presence of less neointimal hyperplasia.[4] On the basis of the porosity and demand of the blood flow by vessels, side-branches

or perforating arteries can be protected if the stenotic area generated by the hybrid stents is less than 50%.[23]

In this study, the patency of all the side-branches was maintained, along with the occlusion of the aneurysms, although the side-branches were not as narrow as the intracranial perforators. If the use of hybrid stents for aneurysmal occlusion is planned, the number of associated perforators should be less to ensure safety.

In the examined aneurysmal model using stent grafts, all small side-branches of the patent artery were patent without significant stenosis. Thus, our hybrid stent represents a step forward in the clinical management of aneurysms.

Conclusion

The long-term results obtained in an animal model indicate that hybrid stents may be effective for aneurysm repair while maintaining side-branch patency.

Acknowledgements

We appreciate the kindness of Shuzoh Yamashita, PhD, President of Japan Stent Technology Co. Ltd, in supplying the MoMo stent for our hybrid stents.

Abbreviations

CCA	Common carotid artery
ICA	Internal carotid artery
BCA	Brachiocephalic artery
SA	Subclavian artery
VA	Vertebral artery
PCoA	Posterior communicating artery
OPhA	Ophthalmic artery
AChA	anterior choroidal artery
PICA	Posterior inferior cerebellar artery
PTCA	Percutaneous transluminal coronal angioplasty
DSA	Digital subtraction angioplasty
H & E	stain Hematoxylin and eosin stain

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Preparation of an autologous heart valve with a stent (stent-biovalve) using the stent eversion method

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Abstract: We designed a novel method for constructing an autologous heart valve with a stent, called a stent-biovalve. In constructing completely autologous heart valves, named biovalves, which used in-body tissue architecture technology, tissues for leaflets were formed via ingrowths into narrow apertures in the preparation molds, frequently leading to delayed or incomplete biovalve preparation. In this technique, self-expandable nitinol stents after everting were mounted on an acrylic column-shaped part and partially covered with an acrylic cylinder-shaped part with three slits. This assembled mold was placed into subcutaneous abdominal pouches in beagles or goats for 4 weeks. Upon removing the acrylic parts after harvesting and trimming of capsulated tissues, a tubular hollow structure with three pocket-flaps of membranous tissue rigidly fixed to the stent's outer surface was obtained. Then, the stent was turned inside out to the original form, thus moving the pocket-flaps from outside to the inside. Stent-biovalves with a sufficient coaptation area were thus obtained with little tissue damage in all cases. The valve opened smoothly, and high aperture ratio was noted. This novel technique was thus highly effective in constructing a robust, completely autologous stent-biovalve with adequate valve function. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 102B: 1038–1045, 2014.

Key Words: heart valve, autologous tissue, stent, biovalve

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INTRODUCTION

Aortic valve disease is one of the most major heart diseases in humans. Once symptoms develop, the average survival of patients with aortic stenosis is reduced to <5 years.[1–3] Patients with severe valvular insufficiency or stenosis typically require valve repair or valve replacement. Surgery for end-stage valvular heart disease consists of two basic alternatives, mechanical and biological prostheses, both of which have significant limitations.[4] While mechanical valves have a functional life span of at least 25 years, they are associated with the need for life-long anticoagulation treatment and the concomitant risks of thromboembolism and bleeding. Biological prostheses generally have better hemodynamic characteristics and do not require long-term aniticoagulation therapies, but are associated with progressive tissue deterioration. Since surgical valve replacement is a highly invasive surgery involving thoracotomy and cardiopulmonary bypass, elderly patients and those with extensive comorbidities cannot undergo this surgery. Since 2002,[5] the less invasive transcatheter aortic valve implantation (TAVI) has been introduced for inoperable and high-risk patients. Recently, with technological advancements, the clinical application of TAVI has extended to intermediate-risk patients.[6] TAVI is expected to evolve further and become more commonly used in the future. However, using a bioprosthetic valve for TAVI has certain disadvantages, since it undergoes progressive degeneration and calcification as it contains no living cells.[7,8]

To overcome these limitations, living heart valves—created by tissue engineering—have been under development. Some heart valves created by tissue engineering have been successfully implanted in animals.[9,10] However, these valves require complicated cell management protocols with cell culture in bioreactors under strictly sterile conditions; this procedure is time-consuming and expensive.

We have previously developed autologous prosthetic tissues by using the "in-body tissue architecture (IBTA)" technology, which is a novel and practical approach for regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in living bodies.[11] This

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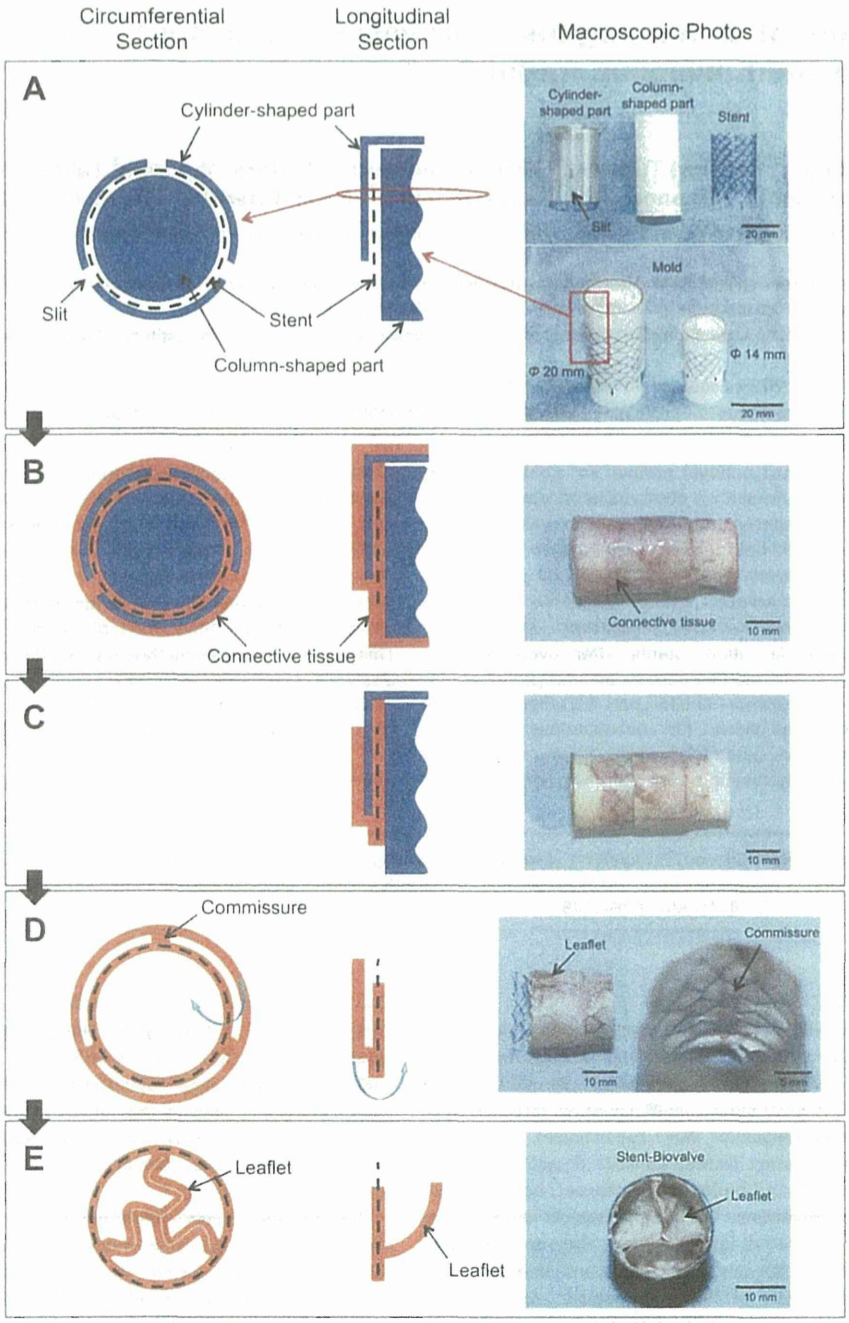

FIGURE 1. Preparation process of the stent-biovalve using the stent eversion method. A specially designed cylinder-shaped part, column-shaped part, and self-expandable nitinol stent (diameter, 14 or 20 mm) were assembled to prepare molds for stent-biovalves (A). After 4 weeks of *in vivo* mold placement, the implants were completely encapsulated with robust connective tissue (B). After trimming the capsulated tissue, the three leaflet parts were obtained (C). The cylinder and column parts were removed to create a patent hollow tube partially fixed with the stent (D). Each stent was iced and turned inside out again (E). The three pocket-flaps that were outside the stent now formed a tri-leaflet valve on the inner surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

technology involves the use of living bodies as a reactor, and does not need expensive facilities or complicated manipulations. We have reported the construction of completely autologous trileaflet heart valves, named biovalves, prepared using this technology,[11–15] which may resolve the abovementioned problems encountered with

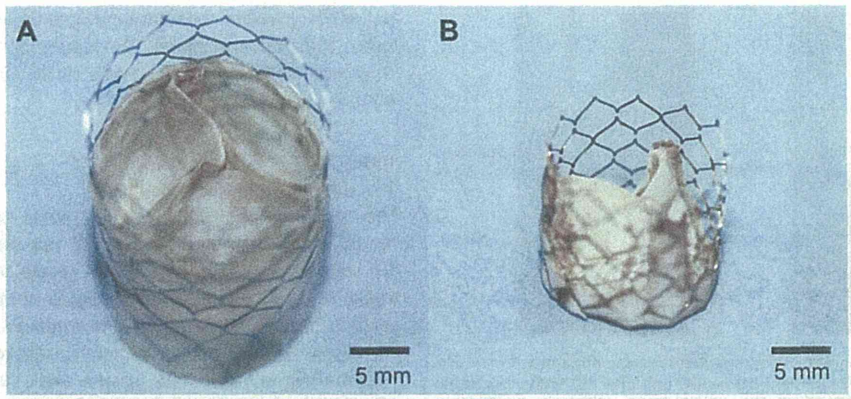

FIGURE 2. The obtained stent-biovalves with diameter of 20 mm (A) and 14 mm (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bioprosthetic valves. In fact, biovalves have been demonstrated to have excellent valve function and histological changes when implanted as a pulmonary valve in a beagle model[16] and an aortic valve in a goat model.[17] In these biovalves, leaflet formation occurs by tissue ingrowth into narrow apertures in the preparation molds from the connective tissues surrounding the molds. During the encapsulation process, the conduit portion of the biovalve is completely formed within ~4 weeks of embedding, similar to the biotubes, which are autologous vascular grafts from IBTA. However, because tissue migration into a narrow aperture is slow in general, leaflet formation in the biovalve required a longer time than that required for conduit formation.

In this study, to further improve the biovalve design and to adapt it for transcatheter valve replacement, we attempted to combine the biovalve design with a self-expandable nitinol stent to construct a "stent-biovalve." The preparation mold was designed based on a novel concept, in which the leaflet tissue was formed on the outer side of the mold; the trileaflet-shaped valve was obtained by finally everting the stent such that the leaflet tissue existed inside surface of the stent. The valvular function of the stent-biovalve was examined by using an *in vitro* circulation circuit.

MATERIALS AND METHODS

Animal studies

Studies were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) under a protocol approved by the National Cerebral and Cardiovascular Center Research Institute Committee (No. 12002).

Mold assembling

Two kind of stents used were self-expandable (diameter- = 14 mm; length = 15 mm or diameter = 20 mm; length- = 30 mm), obtained from shape rememory of E-LUMINEXX (diameter = 12 mm; length = 100 mm; Bard, Karlsruhe, Germany) by Piolax Medical Devices (Yokohama, Japan) and cutting.

The mold for the stent-biovalve was obtained by assembling the stent, a specially designed column-shaped acrylic part (outer diameter = 14 mm; length = 32 mm for 14 mm-sized stent, and outer diameter = 20 mm; length = 46 mm for 20 mm-sized stent), and cylinder-shaped acrylic part (outer diameter = 17 mm; length = 18 mm for 14 mm-sized stent, and outer diameter = 23 mm; length = 34 mm for 20 mm-sized stent) with three slits (width = 1 mm; length = 10 mm for 14 mm-sized stent, and width = 1 mm; length = 15 mm for 20 mm-sized stent) [Figure 1(A)]. All acrylic parts were prepared using a 3D printer (CONNEX 260, Objet, Rehovot, Israel). After gently everting the stent in ice water, it was mounted on the column-shaped acrylic part and then covered with the cylinder-shaped part for the final mold.

Preparation of stent-biovalves

A beagle dog (age: 1 year; body weight: 10 kg) under general anesthesia induced by intramuscular injection of ketamine (20 mg/kg), or a goat (age: 1 year; body weight: 50 kg) under general anesthesia induced with 10 mg/kg of ketamine and maintained with 1–3% isoflurane was used for the stent-biovalve preparation. After 4 weeks of mold embedding in the abdominal subcutaneous pouches of each animal, the implants, which were completely encapsulated with connective tissue, were harvested [Figure 1(B)]. The fragile, irregular, and redundant tissues around the developed tubular tissue were gently cut, and three leaflet parts were obtained by trimming the capsulated tissue [Figure 1(C)]. The acrylic cylinder- and column-shaped parts were removed. The stent, now embedded in connective tissue, also showed three flaps of membranous connective tissue on its outer surface [Figure 1(D)]. The stent was then everted to its original form in ice water to obtain the stent-biovalve, with a tri-leaflet valve on its inner surface [Figure 1(E)]. The stent-biovalves with diameter of 20 mm [Figure 2(A)] were prepared from goats and those of 14 mm [Figure 2(B)] were from beagles.

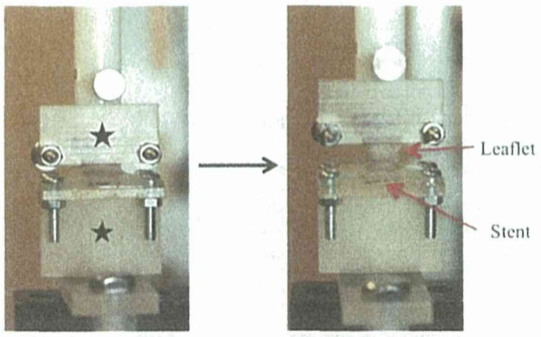

FIGURE 3. Photographs of sample folder in the apparatus for connective strength measurement before (A) and after (B) stretching. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Histological evaluation

The leaflets of the stent-biovalve were fixed in 10% formalin solution and embedded in paraffin. Then leaflet sections were cut into pieces 3–5 μm thick for hematoxyline and eosin staining or masson's trichrome stain. The wall thickness of the leaflets was measured by microscopic observation of the sections.

Measurement of mechanical properties

The burst strength of the leaflet tissues of stent-biovalve was determined by using a specially designed apparatus. The specimens were fixed on a sample holder with a hole (diameter = 2 mm) at its center. Saline solution was introduced into this apparatus at a rate of 50 mmHg/s. The burst strength was determined by measuring the water pressure at the instant of the tissue rupture using a pressure transducer (N5901; Nihon Denki Sanei, Tokyo, Japan).

The elastic modulus of the biotubes was examined using a custom designed tensile tester. Tubular samples were cut circumferentially and opened. Tissue specimens, 10 × 10 mm^2, were tested under humid conditions. The load was recorded until the samples ruptured, with a tissue-extension rate of 0.05 mm/s. Elastic modulus values were obtained from the maximum slope of the deformation-force relationships.

Measurement of the connective strength between leaflet and stent of the stent-biovalve was performed by use of a uniaxial tensile-testing apparatus (Rheoner II; Yamaden, Tokyo, Japan) [Figure 3]. The connective strength between native aortic valve and conduit of goat were also measured in same way. Each sample was fixed in a sample folder that was specially designed by use of a 3D printer (Projet HD3000; 3D Systems, Rock Hill, SC). The testing speed was 0.05 mm/s until failure, that is, tissue rupture. Ultimate tensile strength was calculated from the stress-strain curves.

In vitro valve function

Valve function was examined using a pulsatile circulation circuit (LaboHeart NCVC, IWAKI; working fluid, 0.9% saline; mean arterial pressure, 100 mmHg; mean flow rate, 5–6 L/min, Figure 4). The flow rate, left ventricular pressure, and aortic pressure were measured using an ultrasonic flow meter and pressure meter. The regurgitant ratio and mean flow rate at every 10 bpm from 70 to 120 pulsatile rates were evaluated.

RESULTS

Preparation of stent-biovalves

The two different sized assembled molds [outer diameter of stents, 14 or 20 mm; Figure 1(A)] that were embedded in the subcutaneous pouches of the beagle or the goat for 4 weeks showed complete encapsulation with autologous connective tissue [Figure 1(B)]. The implants could be easily harvested because the developed capsulated tissues and the surrounding subcutaneous tissues were connected only by very fragile, irregular, and redundant tissues, which could be easily removed. The capsulated tissues were dissected to remain the tissue for the leaflets [Figure 1(C)]. The molds could be smoothly removed from both ends of the implant because there was no adhesion between the molds and the tissues covering the stent [Figure 1(D)]. The leaflet tissues were strongly fixed at the three commissures. The stents were iced and then inverted inside out. The tissue flaps, which originally existed outside the stent, were thereby converted to inner leaflets; the stent-biovalves were thus completely prepared [Figure 1(E)]. During the inversion, no or little damage occurred to the leaflet tissues and to the connecting tissues between the leaflet and the stent. Two sized stent-biovalves with a sufficient coaptation area were thus obtained with outer diameter of 14 mm from beagles or 20 mm from goats [Figure 2]. The success rate of the stent-biovalve preparation was 100% (8/8) for each size.

The histological photographs showed that the valve leaflets of the stent-biovalve mainly consisted of collagen fibers [Figure 5]. The wall thickness of the valve leaflet was 285 ± 96.2 μm.

Mechanical properties

The burst strength of the leaflets of the stent-biovalves was over 7600 mmHg, which was closed to that of aortic valve leaflets (6200 ± 1400 mmHg).[16] Elastic modulus of the leaflets of the stent-biovalve was 2.6 ± 1.1 MPa, whereas that of goat aortic valve leaflets was 1.1 ± 0.4 MPa. To evaluate the

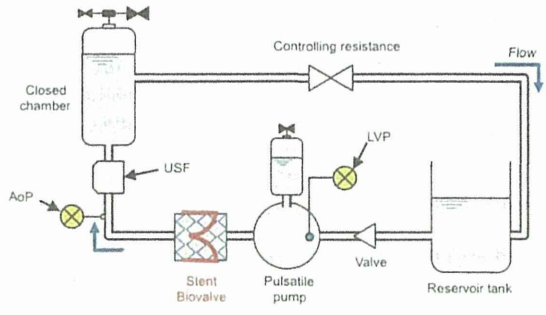

FIGURE 4. A pulsatile circulation circuit model designed for the evaluation of valve function. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

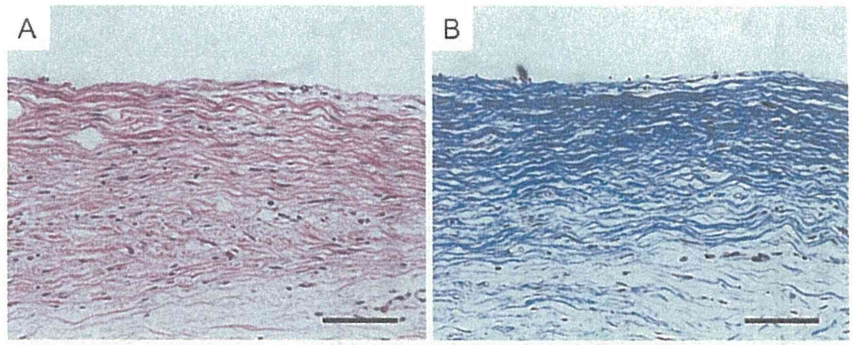

FIGURE 5. Histology of the leaflet of stent-biovalve stained with Hematoxylin and eosin staining (A) and Masson's and trichrome staining (B). bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

connective strength between biovalve leaflet and stent, the tensile strength of the biovalve leaflet and stent was measured by using specially designed sample folder [Figure 5]. The connective strength between biovalve leaflets and stent was comparable to between aortic valve and conduit of goats. The mean values of ultimate tensile strength in each sample were as follows, biovalve leaflet-stent: 833.8 ± 215.5 gf; aortic valve-conduit of goat: 949.7 ± 186.1 gf.

In vitro valve function

The movement of the leaflets in a pulsatile flow circuit [Figure 4] was examined using videography. The stent-biovalve leaflets closed rapidly and tightly in synchronization with the backward flow in the diastolic phase. In the transition phase of the flow direction, the valve opened smoothly and the aperture ratio of the valve was 89% [Figure 6(A)], and coaptation of valve leaflets was optimal [Figure 6(B)].

Figure 6(C) shows the flow rate waveforms of the stent-biovalve at 70 bpm. Regurgitation in the diastolic phase was almost completely prevented. The mean flow rate was ~5–6 L/min [Figure 7(A)], and the regurgitation ratio was ~4% for each heart rate tested [Figure 7(B)].

DISCUSSION

Here, we report the successful development of novel construction method for a stent-biovalve with robust valve leaflets and favorable *in vitro* function. In the previously reported design for biovalves, the molds were designed such that valve leaflets were formed by connective tissue penetrating the apertures in the preparation molds. However, such tissue ingrowth was slow; the formation of the valve leaflets therefore required a long duration and was not always robust.

In this technique, the structure of the new molds for the stent-biovalve ensured successful formation of three valve leaflets, with broad flaps available. The key point of this mold design was that the outer circumferential connective tissue was used to form each valve leaflet. Typically, in a trileaflet valve with a diameter of 14 mm, the horizontal leaflet length for adequate coaptation in the closed valve is 14 mm. Using this method, the leaflet was formed along the acrylic cylinder-shaped part, which had a length of 17 mm, on the outer side of the stent that had a diameter of 14 mm. Thus, the formed leaflet had a length of 17 mm, which is ~1.2 times longer than that required for definitive coaptation. Since the area of the leaflet tissues was sufficient large, an extremely low regurgitation rate (~4%) was noted when testing *in vitro* valvular function, much lower than that noted for the previous biovalve (type IV, regurgitation rate 20%).

Moreover, since the valve leaflets were technically formed in the opening state, each valve leaflet opened smoothly in the systolic phase, resulting in a high aperture ratio of 89%. In addition, the leaflet tissues were very

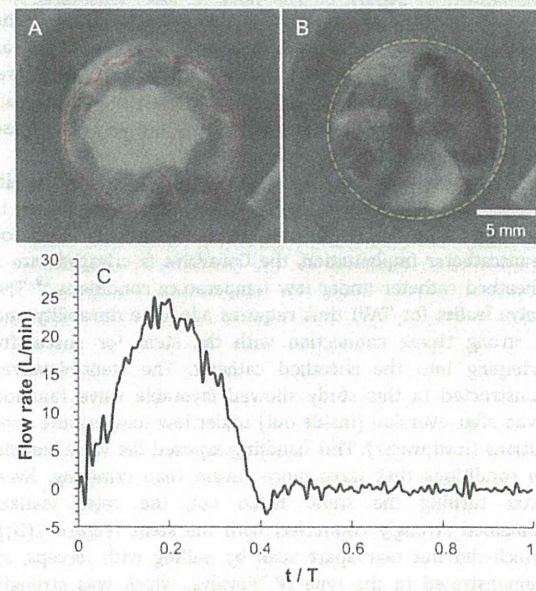

FIGURE 6. Macroscopic photos of the opening (A) and closing (B) form of the stent-biovalve in the circuit as shown in Figure 3. The pulsatile flow was 70 bpm, and the mean flow rate was 5–6 L/min. The aperture ratio was 89%. Pulsatile flow waveform in a single cycle of the stent-biovalve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

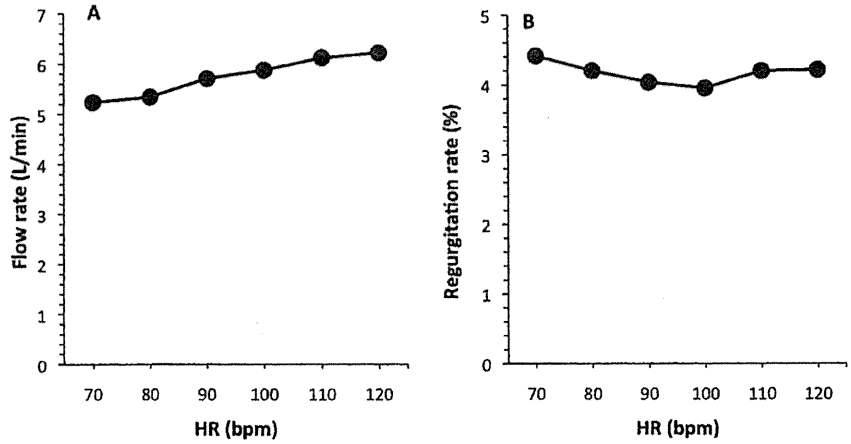

FIGURE 7. Mean flow rate (A) and regurgitation rate (B) at every 10 bpm from 70 to 120 bpm.

robust because the tissues were originally formed as a conduit. The burst strength of the leaflets of the stent-biovalve was over 7600 mmHg, which was about 2 times larger than that of canine pulmonary valve leaflets (3600 ± 780 mmHg) and over that of aortic valve leaflets (6200 ± 1400 mmHg).[16] In addition, elastic modulus of the leaflets of the stent-biovalve was 2.6 ± 1.1 MPa, which was over 2 times larger than that of goat aortic valve leaflets (1.1 ± 0.4 MPa). The cells in the leaflet tissue of the stent-biovalve were rare as shown in Figure 5. Therefore, it was considered that there was little influence of cells in the biomechanics. The connective strength between biovalve leaflet and stent was comparable to native aortic valve and conduit. Therefore, we believe strongly that the robust properties of valve leaflets of the stent-biovalve were demonstrated by these mechanical results.

To facilitate the use of the stent-biovalve in TAVI, a self-expandable nitinol stent was chosen in this study, which is typically used for TAVI (CoreValve, Medtronic, MN). For transcatheter implantation, the CoreValve is crimped into a sheathed catheter under low temperature conditions.[18] The valve leaflet for TAVI thus requires adequate durability and a strong tissue connection with the stent for successful crimping into the sheathed catheter. The stent-biovalves constructed in this study showed favorable valve function even after eversion (inside out) under low temperature conditions (iced water). This handling exposed the valve leaflets to conditions that were more severe than crimping. Even after turning the stent inside out, the valve leaflets remained strongly connected with the stent [Figure 1(D)], which did not tear apart even by pulling with forceps, as demonstrated in the type IV biovalve, which was strongly attached to a scaffold material.[14] Therefore, it is highly expected that the valve leaflets of the stent-biovalve would have sufficient durability and a robust tissue connection with the stent for crimping into a sheathed catheter for TAVI.

Because the biovalve is an engineered tissue, it will have to maintain its function in living bodies, and the living body would be the best environment for testing the biovalve. Therefore, we believe that there is little value in examining the long-term durability of the biovalve in an *in vitro* system. However, in separate study, we evaluated the durability of the biovalve in *in-vitro* pulsatile flow of saline.[19] Even after biovalve pulsated more than four million times (heart rate = 70 bpm; mean flow rate = 5.0 L/min; mean aortic pressure = 92 mm Hg), stable continuous operation was possible without excessive reduction of the flow rate or bursting. However, in our recent report, postoperative echocardiography showed smooth movement of the leaflets of the biovalve with little regurgitation under systemic circulation (2.6 ± 1.1 L/min) at least for 2 months of implantation to goat in an apico-aortic bypass model.[17] Therefore, we believe that the stent-biovalve has similar excellent durability in *in vitro* and *in vivo*.

Recently, several studies reported that tissue engineered valved stents were successfully implanted as pulmonic valve *in vivo*.[20–23] These materials may overcome the limitations of bioprosthetic heart valve prostheses and may be expected further evolution to be applied to clinical use. However, these tissue-engineered valved stents require complicated cell management protocols with cell culture under strictly sterile conditions, and also require decellularized allografts or synthetic scaffold materials to seed autologous cells. In this study, autologous heart valve with stent (stent-biovalve) showed favorable valve function, without expensive facilities or complicated manipulations. Stent-biovalve may overcome the limitations of bioprosthetic heart valve prostheses and have the advantage of other tissue engineered valved stents.

In this study, the pulsatile circuit was designed for actual human aortic valve conditions (mean flow = 5 L/min; mean pressure = 100 mmHg; heart rate = 70–100 bpm); however, saline solution was selected as a working fluid. The kinetic viscosity of blood is 4.4×10^{-6} m^2/s, which is

~4 times that of saline solution (1.0×10^{-6} m^2/s). Thus, our circuit did not approximate the viscosity of blood; however, it was considered that the present design achieved an adequate performance of valvular function for a future animal implantation study. We do not have any additional data on the biomechanics of the tissue affected after long-term stress. However, this is our near future research topics. We are now planning implantation study of the stent-biovalve to beagle dogs. However, we previously reported that in the tissue of the biotube, which was also obtained by IBTA technology from rod molds, the elastic modulus value of the biotube after implantation was 2× higher than that of the native arteries. The biotube after implantation acquired further robust property of maintenance of the preimplantation elongation ability.

Based on the American College of Cardiology/American Heart Association valvular guidelines 2006,[2] the regurgitant fraction of the present stent-biovalve was much lower than the "mild" classification, in fact being present at "trace" levels. Yare et al. reported that 75% of bioprosthetic valves used for TAVI showed trace to mild aortic regurgitation (AR) after implantation; however, such AR did not affect LV structure and function.[24] Previous biovalves were demonstrated to have favorable valve function and histological changes when used as heart valves in a beagle model[14]; further, biotubes created by IBTA technology regenerated arteries within 3 months of implantation.[25] Therefore, although this evaluation of the stent-biovalve was performed *in vitro*, it is expected that this stent-biovalve with autologous valve leaflets will show more favorable function *in vivo* than *in vitro*. We expect that this stent-biovalve could eventually be applied for TAVI, with favorable valve function *in vivo*.

CONCLUSION

We successfully developed a novel method for efficient construction of a robust, completely autologous heart valve eversion of a self-expandable stent, and named the resulting product the stent-biovalve. These stent-biovalves were obtained after only 4 weeks of subcutaneous embedding in a beagle dog or a goat. Owing to the large and robust leaflet tissues that were developed in the open-form position, excellent *in vitro* valve function was achieved. Future studies are expected to focus on the implantation of this stent-biovalve in animal models to confirm chronic valve function and durability *in vivo*.

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In Vitro Evaluation of a Novel Autologous Aortic Valve (Biovalve) With a Pulsatile Circulation Circuit

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Abstract: We have used in-body tissue architecture technology to develop an autologous valved conduit with intact sinuses of Valsalva (biovalve). In this study, we fabricated three different forms of biovalves and evaluated their function in vitro using a mock circulation model to determine the optimal biovalve form for aortic valve replacement. A cylindrical mold for biovalve organization was placed in a dorsal subcutaneous pouch of a goat, and the implant that was encapsulated with connective tissue was extracted 2 months later. The cylindrical mold was removed to obtain the biovalve (16 mm inside diameter) that consisted of pure connective tissue. The biovalve was connected to a pulsatile mock circulation system in the aortic valve position. The function of the three biovalves (biovalve A: normal leaflets with the sinuses of Valsalva; biovalve B: extended leaflets with the sinuses of Valsalva; biovalve C: extended leaflets without the sinuses of Valsalva) was examined under pulsatile flow conditions using saline. In addition, the mock circuit was operated continuously for 40 days to evaluate the durability of biovalve C. The regurgitation rate (expressed as a percent of the mean aortic flow rate during diastole) was 46% for biovalve A but only 3% for biovalves B and C. The durability test demonstrated that even after biovalve C pulsated more than four million times (heart rate, 70 bpm; mean flow rate, 5.0 L/min; mean aortic pressure, 92 mm Hg), stable continuous operation was possible without excessive reduction of the flow rate or bursting. The developed biovalve demonstrated good function and durability in this initial in vitro study. **Key Words:** Heart valve—Biovalve—Pulsatile circulation circuit—Autologous tissue—Aortic valve—Tissue engineering.

Prosthetic valve replacement is an essential treatment for severe valvular heart disease (1). Mechanical valves and biological valves provide excellent valvular function and are currently implanted to treat serious valve disease. However, both of these valves have limitations. Although mechanical valves are durable, anticoagulants such as warfarin are required to prevent thrombosis. Although biological valves, which are usually heterologous (made of bovine pericardia or porcine valves), are less thrombogenic, they may undergo calcification and structural deterioration over time.

Recently, heart valve prostheses have been developed using tissue engineering technology, and advantages of these tissue-engineered valves would likely include nonthrombogenicity, infection resistance, and cellular viability. If tissue-engineered valves are implanted in children, there is the possibility of growth, repair, and remodeling as a child matures, thus eliminating the repetitive surgeries typically required to implant conventional mechanical or biological valves as body size increases (2). We have been developing autologous prosthetic tissues to function as heart valves or grafts using "in-body tissue architecture technology," which is a novel concept in regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in living bodies (3–7). This technology involves the use of living bodies as reactors, and its advantages are that it is simple, safe, and cost-effective. In previous studies, we used this technology to develop an autologous valved conduit with the sinuses of Valsalva and named this new valve the "biovalve" (5–7). The biovalve was developed using a canine model , and its function was determined when it was

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used to replace the pulmonary valve (5). Smooth opening and rapid closing of the leaflets were confirmed in vitro. In addition, the biovalve was implanted in beagles to replace the pulmonary valve and was able to maintain normal hemodynamic function for approximately 3 months. To further develop the biovalve, our goal is to fabricate the biovalve using large animals and to engineer a valve that can replace the aortic valve. Recently, biovalves were fabricated using goats and showed smooth movement of the leaflets with little regurgitation when they were implanted in a specially designed apico-aortic bypass circuit in a goat model (7). Further evaluation of valvular function and an endurance test is needed to determine the optimal form of the biovalve that should be used to replace the aortic valve.

In this study, we fabricated three different forms of biovalves and evaluated their function and durability using a mock circulation model in vitro.

MATERIALS AND METHODS

Preparation of biovalves

Figure 1 shows the process of preparation of the biovalves. A specially designed cylindrical mold for biovalve organization is shown in Fig. 1a. The cylindrical mold was constructed from seven plastic parts. First, three small hemisphere-like parts with projections resembling the protrusion of the sinuses of Valsalva were fastened between two tube-like parts (diameter, 16 mm) that served as the conduit, with a small aperture of 0.5 mm. Second, the combined parts were fixed with a rod-like center part and a latch part to obtain the final mold. The mold was designed such that the leaflets were separated from each other in the open form and connective tissue entered through its aperture (8). We chose goats as an experimental animal in order to evaluate the biovalve in an environment similar to the valve size and hemodynamics in humans. Several cylindrical molds were placed in a dorsal subcutaneous pouch in 10 goats (age, 1–2 years; body weight, 40–50 kg) under general anesthesia induced with 10 mg/kg of ketamine and maintained with 1–3% isoflurane (Fig. 1b). The implant which was encapsulated with connective tissue was extracted 2 months later (Fig. 1c). The cylindrical mold was removed from the implant to obtain the biovalve that consisted of pure connective tissue (Fig. 1d,e). The biovalve was stored at −30°C in 70% ethanol. As the storage in 70% ethanol is not fixation but dehydration of the tissue, the suppleness of the biovalves which were rehydrated recovers. The biovalve including the leaflets, conduit, and sinus of Valsalva was mainly composed of collagen-rich tissue with fibroblasts (Fig. 1f,g). There were few elastic fibers and vascular cells. The elongation at the break, indicative of tissue extensibility, was obtained from the stress-strain curves which were measured using a tensile tester (7). The elongation at the break in the leaflet and conduit of the biovalve were 66.7 ± 8.3 and 68.7 ± 6.9%. Here, the elongation at the break in the leaflet and conduit of the native valve were 108.3 ± 12.5 and 64.6 ± 10.4%. Although elongation at the break in the leaflet of the biovalve was about a

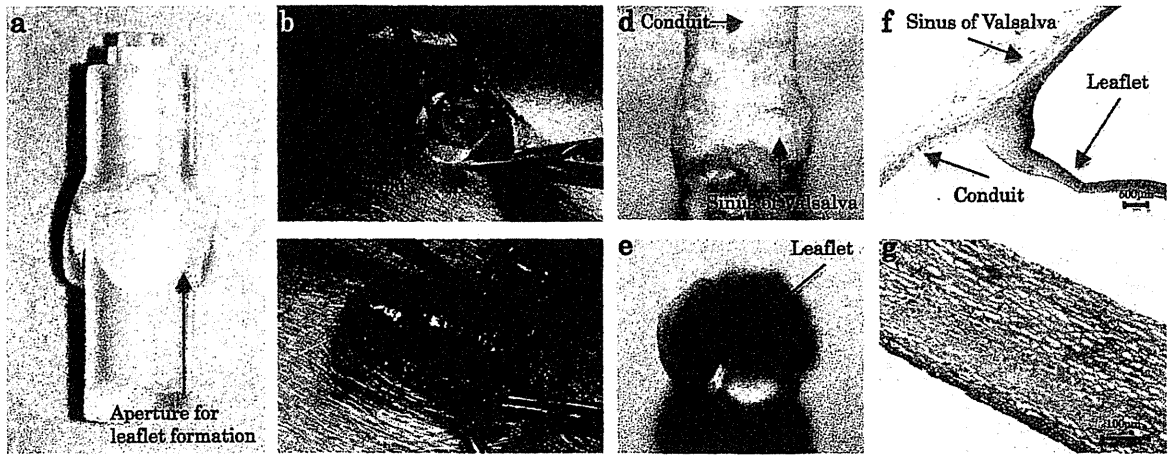

FIG. 1. Preparation process of the biovalve. (a) A specially designed cylindrical mold was constructed from seven plastic parts to act as a scaffold for the biovalves. (b) The mold was placed in the dorsal subcutaneous pouch of a goat. (c) The implant which was encapsulated with connective tissue was extracted 2 months later. (d, e) The cylinder mold was removed from the implant, and the biovalve completely produced only by connective tissue was obtained. (f) The cross section of the whole biovalve stained with hematoxylin and eosin. (g) The extended cross section of the leaflet stained with hematoxylin and eosin.

TABLE 1. *Biovalve measurements*

Outer diameter of conduit (mm)	17
Inner diameter of conduit (mm)	16
Maximum diameter at the sinuses of Valsalva (mm)	19
Tissue thickness (mm)	0.5
Length (mm)	55

half of the native value, the biovalve had suppleness similar to the native valve. Although these data were measured after storage in 70% ethanol, the suppleness of the biovalves was maintained. The dimensions of the biovalve are given in Table 1.

Institutional guidelines for the care and use of laboratory animals were observed. All protocols were reviewed and approved by the Animal Subjects Committee of the National Cerebral and Cardiovascular Center (no. 12002).

Valves evaluated

There were five different valves examined in this study (Fig. 2). Three of the valves were biovalves with slightly different designs (Fig. 2a–e), and two were commercially available valves that were compared with the biovalves. The latter two valves were a biological valve (19 mm, St. Paul, MN, USA) (Fig. 2f) and a mechanical valve (23 mm, Bicarbon Bileaflet Valve, Sorin Biomedica, Saluggia, Italy) (Fig. 2g).

Biovalve A (Fig. 2a) was developed with the sinuses of Valsalva using the same mold as in a previous study (8). The leaflets of this biovalve were designed to conform to the shape of a porcine valve. Each leaflet had an area of 240 mm^2 and a length of 17 mm (Fig. 2d).

Biovalve B (Fig. 2b) was fabricated with leaflets that were 2 mm longer in an axial direction than biovalve A. This was done to improve the operating performance of the biovalve under systemic circulatory conditions. This model also had an outer casing (19 mm inner diameter) that allowed growth of the sinuses of Valsalva in a radial direction. Each leaflet had an area of 290 mm^2 and a length of 19 mm (Fig. 2e).

Biovalve C (Fig. 2c) was the same as biovalve B but without the sinuses of Valsalva to determine whether or not the presence of these sinuses influenced

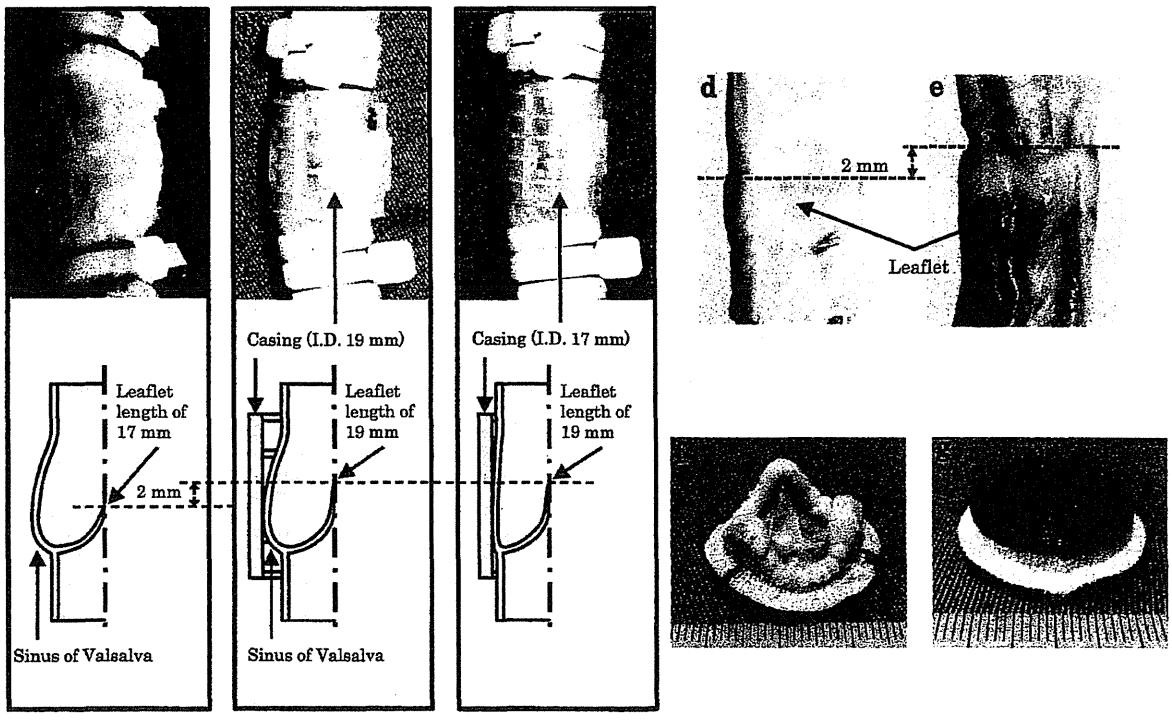

FIG. 2. The valves employed in this study. (a) Biovalve A had a leaflet length of 17 mm. (b) Biovalve B had a leaflet length of 19 mm and had an outside casing (19 mm inner diameter) that was used to prevent the sinuses of Valsalva from extending in a radial direction beyond the design value. (c) Biovalve C had a leaflet length of 19 mm and had an outside casing (17 mm inner diameter) that was used to prevent growth of the sinuses of Valsalva. (d) Interior surface of biovalve A. (e) Interior surface of biovalves B and C. (f) Biological valve (19 mm, Epic Supra Valve, St. Jude Medical, Inc.). (g) Mechanical valve (23 mm, Bicarbon Bileaflet Valve, Sorin Biomedica).

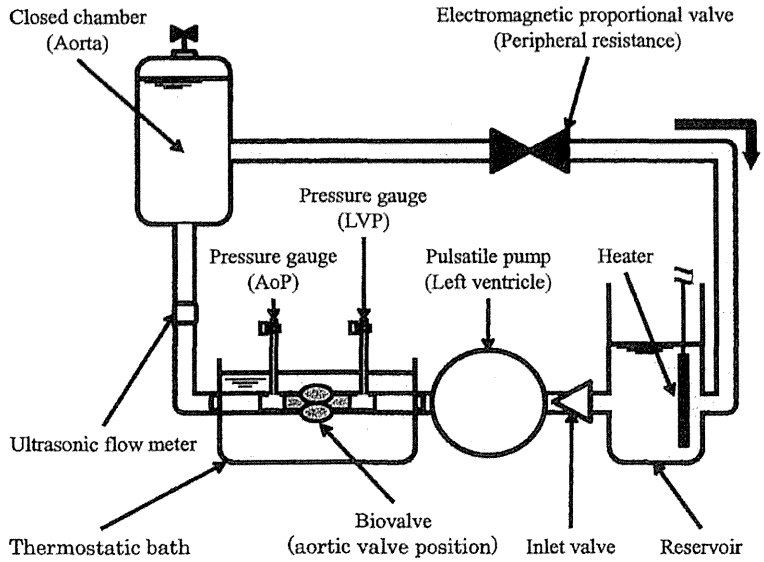

FIG. 3. Schematic drawing of the pulsatile mock circulation system that was used for the evaluation of valvular function and durability.

valvular function. This design included an outer casing (17 mm inner diameter) to prevent any growth of the sinuses of Valsalva.

Evaluation of valvular function

A pulsatile mock circulation system (LaboHeart NCVC, IWAKI Co., Ltd., Tokyo, Japan) (9) was used to simulate the systemic circulation to evaluate the valvular function of the five valves. Figure 3 shows a schematic drawing of the circulation circuit. The pulsatile mock circulation system consisted of the following elements: a pulsatile pump, a closed chamber, a reservoir, and an electromagnetic proportional valve to create fluid resistance. The mock circulation system can simulate various pulsatile conditions by adjusting the heart rate (HR) and stroke volume of the pulsatile pump, the fluid resistance of the electromagnetic proportional valve, and the compliance of the closed chamber. Each of the five valves was connected at the aortic position in the pulsatile mock circulation system. An inlet valve made of synthetic rubber was connected at the mitral valve position. The flow rate was measured using an ultrasonic flow meter (T106, Transonic Systems, Ithaca, NY, USA) attached between the valve and the closed chamber. Pressure at the aortic valve inlet reflected left ventricular pressure (LVP), and pressure at the outlet reflected aortic pressure (AoP). These two pressures were measured using a pressure gauge (PA-500, Nidec Copal Electronics Corporation, Tokyo, Japan). The stroke volume of the pulsatile pump, the compliance of the closed chamber, and fluid resistance were adjusted to maintain an average AoP of 100 ± 20 mm Hg as the HR of the pulsatile pump was varied from 70 to 120 bpm. Then, the flow rate, LVP, and AoP were measured at each HR. The regurgitation rate was calculated from the following equation: regurgitation rate (%) = (mean regurgitation/mean flow rate) $\times$ 100 (1). In this study, the regurgitation was defined as flow rate <0 L/min. The valvular functions of all valves were also measured with the same circuit, stroke volume, fluid resistance, and chamber compliance.

Long-term continuous operation

The circulatory model with biovalve C inserted at the aortic valve position was operated continuously for 40 days to evaluate the durability of the biovalve. The experiment was conducted under fixed conditions, and the pulsatile pump, closed chamber, and fluid resistance were adjusted to produce a mean flow rate of 5 L/min and mean AoP of 100 mm Hg when the HR of the pulsatile pump was set at 70 bpm. Several factors, including valve failure or fracture, valvular insufficiency, and changes in the pressure waveform, were evaluated.

Experimental conditions and data acquisition

Saline (viscosity, 0.001 Pa·s) at 37°C was used as the working fluid and the immersion fluid for all of the biovalves. A heater and a thermostatic bath were used to maintain a constant temperature. In the case of the long-term continuous operation experiment, a corrosion inhibitor (white7-SW, Yuai Kasei, Hyogo,