# Biodegradable polymer with collagen microsponge serves as a new bioengineered cardiovascular prosthesis

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**Objective:** Biodegradable materials with autologous cell seeding have attracted much interest as potential cardiovascular grafts. However, pretreatment of these materials requires a complicated and invasive procedure that carries the risk of infection. To avoid these problems, we sought to develop a biodegradable graft material containing collagen microsponge that would permit the regeneration of autologous vessel tissue. The ability of this material to accelerate in situ cellularization with autologous endothelial and smooth muscle cells was tested with and without precellularization.

**Methods:** Poly(lactic-co-glycolic acid) as a biodegradable scaffold was compounded with collagen microsponge to form a vascular patch material. These poly(lactic-co-glycolic acid)–collagen patches with (n = 10) or without (n = 10) autologous vessel cellularization were used to patch the canine pulmonary artery trunk. Histologic and biochemical assessments were performed 2 and 6 months after the implantation.

**Results:** There was no thrombus formation in either group, and the poly(lactic-co-glycolic acid) scaffold was almost completely absorbed in both groups. Histologic results showed the formation of an endothelial cell monolayer, a parallel alignment of smooth muscle cells, and reconstructed vessel wall with elastin and collagen fibers. The cellular and extracellular components in the patch had increased to levels similar to those in native tissue at 6 months.

**Conclusions:** The poly(lactic-co-glycolic acid)–collagen microsponge patch with and without precellularization showed good histologic findings and durability. This patch shows promise as a bioengineered material for promoting in situ cellularization and the regeneration of autologous tissue in cardiovascular surgery.

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In the history of cardiovascular surgery, glutaraldehyde-fixed xenograft, autologous pericardium, polytetrafluoroethylene, and Dacron have been used as graft material for blood vessel reconstruction or replacement. These materials, however, have well-known limitations, such as thrombogenicity, calcification, shrinkage, and lack of growth potential. The concept of tissue engineering with a biodegradable scaffold was proposed in the 1990s to overcome these problems.[1] This idea has been developed and applied clinically with autologous cell seeding in recent years.[2-6] However, the ex vivo cell-seeding pretreatment (precellularization) is complicated, invasive, and prone to infection. Therefore the development of a biodegradable material that allows efficient in situ cellularization without precellularization is an important strategy. Recently, we

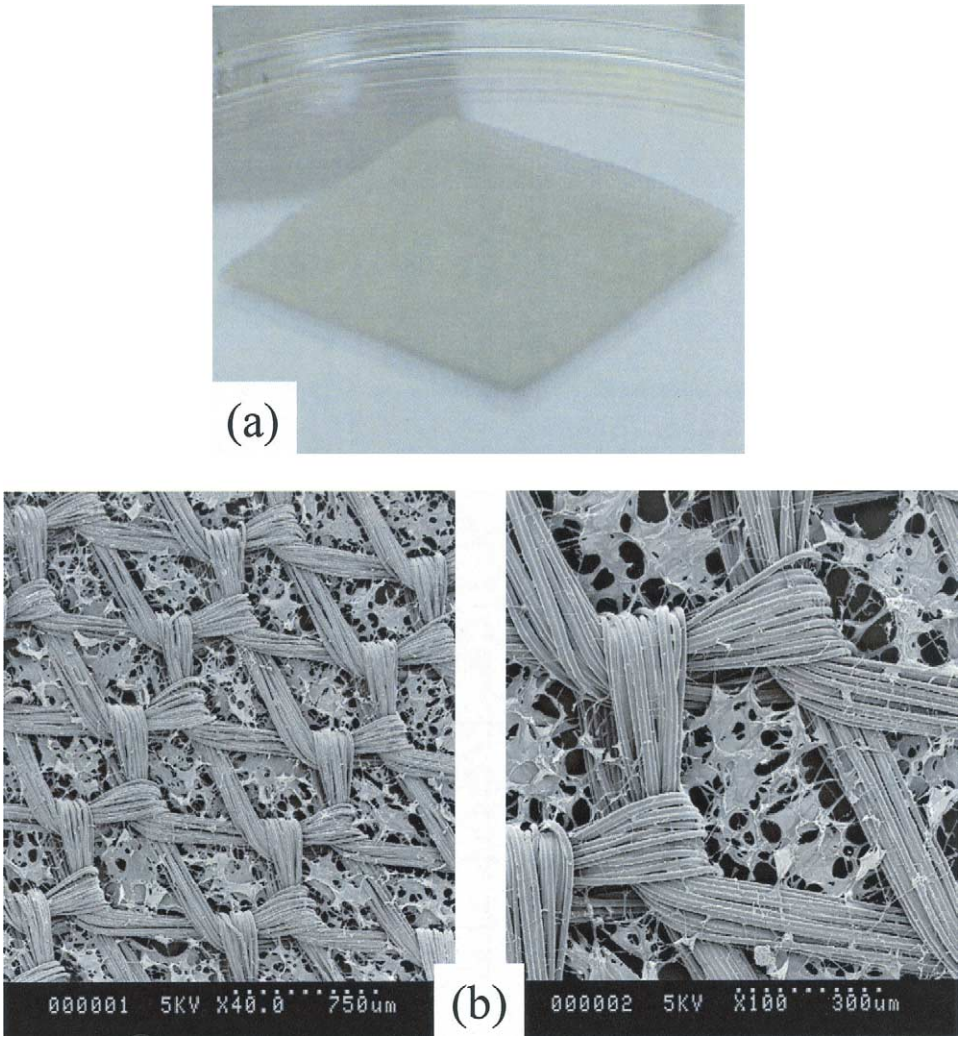


**Figure 1. The PLGA-collagen microsponge patch was 0.6 mm thick (a). Scanning electron microscopy showed the collagen microsponge had a uniformly distributed and interconnected pore structure (pore size of 50-150 $\mu$m; b).**

developed a biodegradable patch by compounding a polymer scaffold with collagen microsponge.

Naturally derived collagen has the potential advantage of interacting with specific cells and is an essential component of the extracellular matrix that supports endothelial and smooth muscle cell (SMC) expansion. Moreover, collagen microsponge is thought to have good cell-attachment properties because of its porous 3-dimensional structure.[7] Considering these advantages, we proposed the feasibility that a bioengineered cardiovascular patch with collagen microsponge might serve as a nonthrombogenic and mechanically durable prosthesis with in situ cellularization.

In the present study we examined the histologic and biochemical characteristics of this patch after its implantation in a dog model for reconstruction of the right ventricular outflow tract by using patches with and without a precellularization step.

## Materials and Methods

### Scaffold Design

The patch was fabricated by compounding collagen microsponge with a biodegradable synthetic polymeric scaffold composed of fibers made from poly(lactic-co-glycolic acid) (PLGA) 90:10 mesh (PLGA-collagen patch). The PLGA mesh was provided by Ethicon, Inc (Somerville, NJ). The techniques for fabricating the PLGA-collagen microsponge have been described previously in detail by Chen and associates.[7] Briefly, the PLGA mesh was immersed in a solution containing type I and type IV collagen (BD Biosciences; type I:IV = 9:1). The collagen-coated mesh was then frozen at −80°C for 12 hours and lyophilized in a vacuum of 0.2 mm Hg for an additional 24 hours to allow the formation of collagen microsponge. The collagen microsponge was further cross-linked by treatment with glutaraldehyde vapor through exposure to a 25% glutaraldehyde aqueous solution at 37°C for 4 hours. The completed patch is shown in Figure 1.

### Cell Isolation, Culture, and Seeding

Segments of the V. saphena magna (3-4 cm) were harvested from 8-month-old beagle dogs. Endothelial cells were obtained by instilling 0.1% collagenase and 0.012% trypsin into the vessel segments. The resulting endothelial cells were cultured in Dulbecco modified Eagle medium (Gibco BRL-Life Technologies) supplemented with 10% fetal bovine serum (Sigma Chemical Co) and 1% penicillin, streptomycin, and amphotericin (Gibco). To obtain medial cells, which are mostly SMCs, the remaining de-endothelialized vessel segments were flushed with 0.07% collagenase and 0.03% elastase (Sigma), and the medial cells were cultured with the same medium. The cell populations were passaged 3 times to obtain enough cells for cell seeding. For seeding, first 8 million medial cells were seeded onto the surface of the PLGA-collagen patch (20 × 15 mm). Twenty-four hours later, 4 million endothelial cells were dripped onto the seeded patch. After 2 days of dynamic culture with stirring at 50 rpm in a humidified incubator, which was done to increase cell-seeding efficiency,[8] the patches were implanted.

### Ex Vivo Study

Two sets of PLGA-collagen patches (10 × 10 mm) made with type I collagen only (n = 5) or type I plus type IV mixed collagen (n = 5) were cultured with seeded endothelial cells and SMCs labeled with a fluorescent cell tracer (PKH26, Sigma) for 3 days. The attachment and expansion areas of these cells were calculated with image analysis software (MacScope, Macintosh) and expressed as the percentage of the visual field.

### In Vivo Study

Two sets of PLGA-collagen patches (20 × 15 mm) prepared with precellularization (P-C[+] group; n = 10) or without precellularization (P-C[−] group; n = 10) were implanted onto the pulmonary trunk of beagle dogs (body weight, approximately 8-10 kg). Anesthesia was induced with an intravenous bolus infusion of 3 mg/kg ketamine and 3 mg/kg sodium barbiturate and maintained by means of inhalation of sevoflurane. The heart was approached through a left anterolateral thoracotomy, entering the chest through the fourth intercostal space. The pulmonary trunk was partially clamped and patched with the PLGA-collagen patch by using 5-0 monofilament running sutures. The patch was explanted 2 (n = 5 in each group) or 6 (n = 5 in each group) months after the implantation. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

### Histologic and Immunohistologic Examination

Explanted tissue specimens were examined with hematoxylin-eosin–, elastica van Gieson–, and von Kossa–stained paraffin or immunostained frozen sections. The antibodies used for immunohistochemistry were monoclonal antibodies to CD31 (clone JC/70A, DAKO) and α-smooth muscle actin (clone HHF35, DAKO), and a polyclonal antibody to von Willebrand factor (factor VIII–related antigen; rabbit, N1505, DAKO). Other antibodies used for immunofluorescence were a monoclonal antibody to collagen type I (clone COL-1, Sigma), and a polyclonal antibody to collagen type IV (rabbit, Rockland).

### Mechanical Strength

The maximal tensile strength of the PLGA-collagen patch was measured before implantation and 2 and 6 months after implantation with a TENSILON mechanical tester (Orientec Co, Ltd). The dimensions of the patches used for the tensile test were 5 × 20 mm, and the patches were pulled to failure at a rate of 10 mm/min. The tension strength was represented by N units (1 N = 1 MPa · measured $mm^2$).

### Biochemical Examination

A 4-hydroxyproline assay was used to measure the collagen content in the explanted patch. The elastin content was quantified by determining the dry weight of the insoluble material after delipidation in acetone–diethyl ether and solubilization in 0.1 N NaOH at 98°C. The DNA content, which reflects the cellular component, was measured with a Hoechst dye assay (Hoechst 33258, Molecular Probes Inc). The amounts of these cellular and extracellular components in the constructs were compared with those of the native pulmonary artery from the same animal and were expressed as a percentage of the native level.

All results were expressed as the mean value ± SD.

## Results

### Ex Vivo Study

The attachment and expansion area of endothelial cells cultured on the PLGA-collagen type I plus IV (55% ± 11%) was higher than on the PLGA-collagen type I (40% ± 6%). The attachment and expansion area of SMCs on the PLGA-collagen type I plus IV (69% ± 10%) was also higher than on the PLGA-collagen type I (40% ± 6%).

### In Vivo Study

***Macroscopic findings.*** On macroscopic inspection, neither group showed thrombus formation on the internal surface of the patch. The size of the patches was unchanged 6 months after implantation (before implantation, 20 × 15 mm; P-C[+], 20.4 ± 2.8 × 14.2 ± 0.8 mm; P-C[−], 19.5 ± 1.3 × 14.3 ± 1.3 mm). There was no intimal thickening after the implantation of patches either with or without precellularization. The thickness of the precellularized patch, untreated patch, and native pulmonary artery were 1.1 ± 0.1, 1.0 ± 0.1, and 1.0 ± 0.1 mm, respectively (Figure 2).

***Histologic examination.*** Microscopic examination of the patch with precellularization 2 months after implantation showed that numerous seeding cells, which were labeled with a fluorescent tracer, remained on the patch (Figure 3). Hematoxylin-eosin staining showed almost complete absorption of the PLGA polymer in both groups 2 months after implantation (Figure 4, *a*). A monolayer of endothelial cells covering the surface of the patch was confirmed by staining with factor VIII and CD31 (Figure 4, *b*). α-Smooth muscle actin staining showed a parallel alignment of SMCs in the wall of the patch in both groups (Figure 4, *c*). Elastica van Gieson staining demonstrated the remodeling of elastin

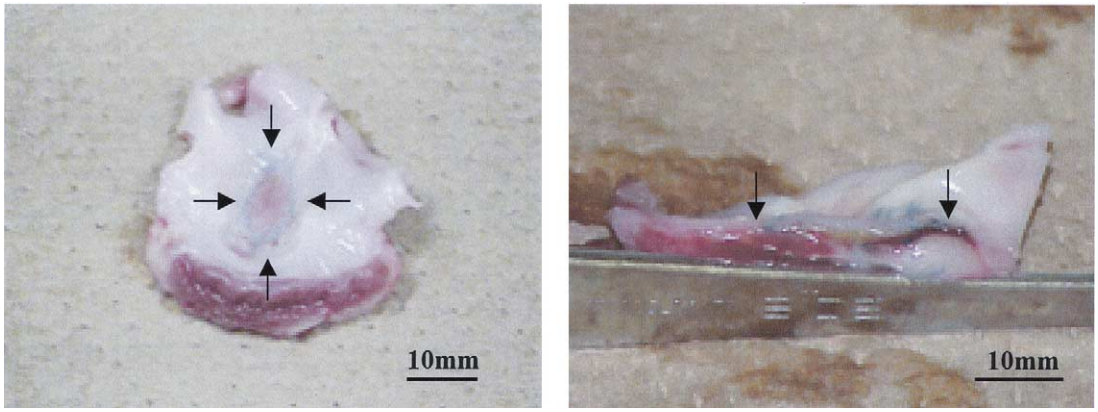


**Figure 2. Macroscopic findings 6 months after implantation. There was no thrombus formation in any case. The *arrows* show the border of the patch and the native pulmonary artery. The appearance of the reconstructed portion was not significantly different from that of the native pulmonary artery.**

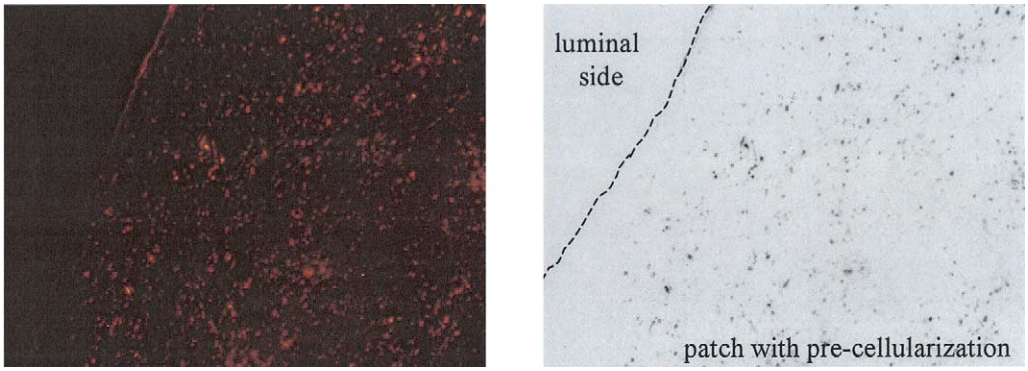


**Figure 3. Microscopic findings of the patch with precellularization after 2 months of implantation showed numerous remaining seeding cells, which were labeled with a fluorescent tracer. (Original magnification 40 ×.)**

fibers in the tissue (Figure 4, *d*). Immunofluorescence staining of collagen showed a similar pattern as that seen in native tissue, with type IV collagen present at the basal membrane (Figure 5). These histologic findings were almost the same in both the grafts with and those without precellularization. No calcification was detected by means of von Kossa staining, and the architecture of the implanted patch was similar to that of the native pulmonary artery wall 6 months after implantation (Figure 6).

***Mechanical strength.*** The mechanical tensile strength of the PLGA-collagen patch before and after implantation was greater than that of the native pulmonary artery (before implantation: 74.8 ± 3.2 N; after 2 months: P-C[+], 14.8 ± 2.6 N; P-C[−], 14.2 ± 4.4 N; after 6 months: P-C[+], 26.8 ± 7.0 N; P-C[−], 28.7 ± 8.1 N; native pulmonary artery: 2.7 ± 0.9 N).

***Biochemical examination.*** The 4-hydroxyproline assay demonstrated that the implanted patch had almost the same collagen content as the native tissue (after 2 months: P-C[+], 127% ± 26%; P-C[−], 110% ± 15%; after 6 months: P-C[+], 135% ± 35%; P-C[−], 117% ± 30% of the collagen content of native pulmonary artery). The elastin content was about 60% that of native tissue at 2 months and increased to a level similar to that of native tissue at 6 months (after 2 months: P-C[+], 51% ± 42%; P-C[−], 54% ± 29%; after 6 months: P-C[+], 91% ± 18%; P-C[−], 90% ± 31%). The DNA assay revealed an increase in DNA in the patches to levels comparable with that of native pulmonary artery at 2 months after implantation (after 2 months: P-C[+], 113% ± 23%; P-C[−], 95% ± 22%; after 6 months: P-C[+], 101% ± 25%; P-C[−], 87% ± 26%).

## Discussion

In this study we evaluated the histologic, mechanical, and biochemical characteristics of the PLGA-collagen microsponge patch with or without precellularization after its implantation into a dog pulmonary artery. PLGA is usually absorbed at 2 months in situ.[9] In agreement with this, all cases in the present study showed the disappearance of the polymeric fiber at 2 months. The histologic findings at 2

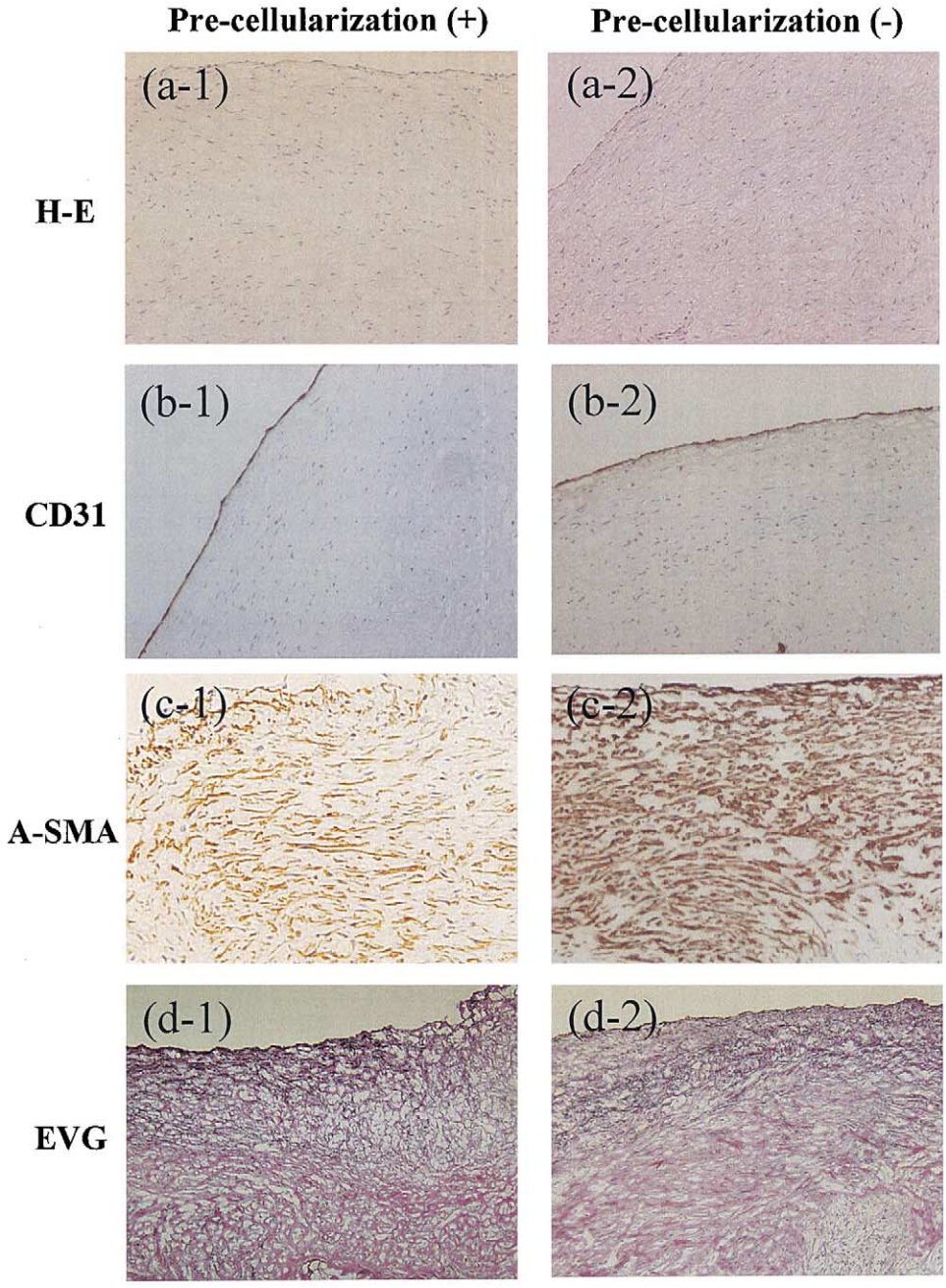


**Figure 4. Histologic findings 2 months after implantation. (Original magnification 100 ×.) Hematoxylin-eosin staining *(H-E)* with (a-1) and without (a-2) precellularization treatment, CD31 staining with (b-1) and without (b-2) precellularization, $\alpha$-smooth muscle actin staining *(A-SMA)* with (c-1) and without (c-2) precellularization, and elastica van Gieson staining *(EVG)* with (d-1) and without (d-2) precellularization are shown. These results show the formation of an endothelial cell monolayer, a parallel alignment of SMCs, and reconstructed vessel wall with elastin and collagen fibers in both groups.**

months after grafting showed good cellularization in both groups. The implanted patches in both groups had sufficient mechanical strength for reconstruction of the vascular wall, possibly because of the addition of fibrous connective tissue resulting from the wound-healing process. We used tensile strength as our biomechanical test, which might not completely reflect the physiologic biomechanical properties of the patch. Nonetheless, the tensile strength at 2 months of

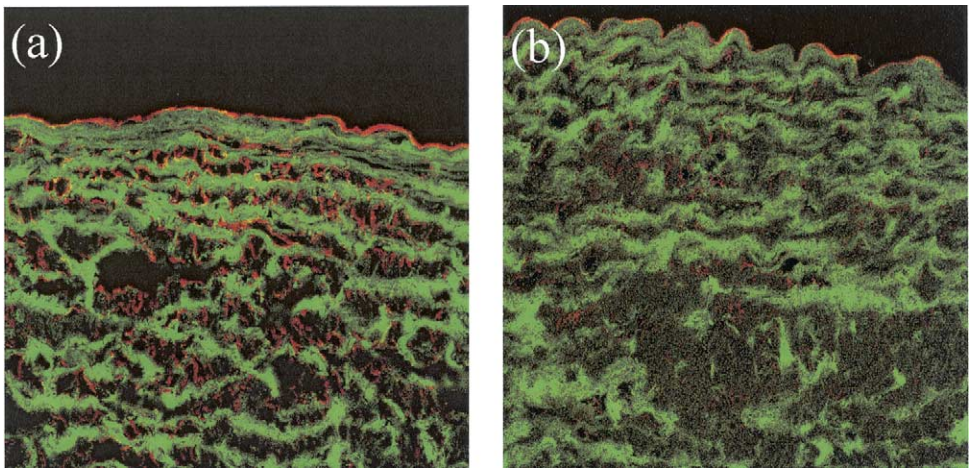


**Figure 5. Immunofluorescence staining of collagen. (Original magnification 100 ×.) Green reveals type I collagen, and red shows type IV collagen. The patch 2 months after implantation without precellularization (a) showed a similar pattern as that seen in the native pulmonary artery (b), with type IV collagen at the basal membrane.**

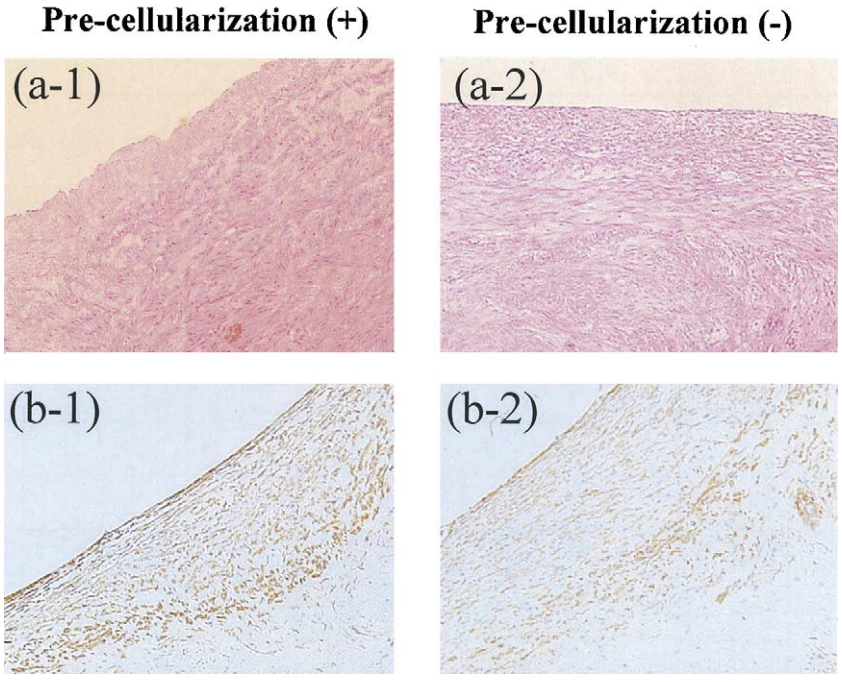


**Figure 6. Hematoxylin-eosin staining (original magnification 40 ×) of the patch 6 months after implantation with (a-1) and without (a-2) precellularization showed a similar architecture to the native pulmonary artery. $\alpha$-Smooth muscle actin staining (original magnification 40 ×) of the patch 6 months after implantation with (b-1) and without (b-2) precellularization showed widely expanded SMCs in the wall of the patch.**

implantation, when the polymer had been absorbed, was comparable with that of native pulmonary artery. There was no sign of rupture in any case up to 6 months after implantation. The percentage of cellular and extracellular components in the patch had increased to levels similar to those in native tissue at 6 months. In our experiments quantifying the collagen content, we could not discriminate between the bovine collagen of the initial patch and the newly generated canine collagen. However, immunofluorescence staining of the collagen showed a pattern similar to that of native tissue, such as the wave form of type I collagen and the presence of type IV collagen at the basal membrane.

The currently available graft materials used in congenital heart surgery have been found to be unsatisfactory when evaluated with respect to long-term results. Furthermore, they are associated with serious problems, such as calcification, peel formation, and a lack of growth potential.[10,11] The concept of tissue engineering with a biodegradable scaffold has been proposed[1] and tested[2-6] both in experimental and clinical situations to overcome these limitations. In previous reports precellularization with autologous cells harvested from the bone marrow, peripheral vessels, or blood was performed before implantation of the grafts. The harvesting and seeding of the cells, however, have the potential to cause complications, such as hemodynamic effects caused by bone marrow aspiration and contamination during the cell culture and seeding steps before implantation.

On the other hand, the collagen microsponges we used in the present study have a porous 3-dimensional structure (pore sizes of 50-150 $\mu$m) that accelerates the cell attachment to the scaffold.[7] The hydrophilicity of the collagen and interconnected pore structure of the microsponge facilitate the attachment and spreading of cells throughout the patch.[12] In a preliminary experiment we found that the PLGA scaffold without the collagen microsponge gave rise to immature cellularization and thrombus formation (data not shown). Another report similarly shows that pulmonary artery replacement with a polymer tube without precellularization resulted in severe thrombus formation that led to pulmonary stenosis.[13] Here we did not study endothelialization earlier than 2 months after implantation. However, the collagen microsponge coating on the polymer scaffold might have a role in preventing the thrombus formation associated with early endothelialization. In the present study there was no thrombus formation on the surface of the patch, even without precellularization. This means that coating the PLGA material with collagen microsponge might have a similar effect as preseeding the PLGA at phases earlier than 2 months.

In addition to precellularization, many strategies have been used to promote cell differentiation on graft materials, such as the application of other extracellular matrix components or growth factors.[14-17] In this study we evaluated using 2 types of collagen, type I and type IV, as an additive for regeneration of the vascular vessel wall. Collagen type IV, which localizes to the basement membranes of vascular tissue, has an important role in cell differentiation.[18] Our findings with ex vivo experiments suggested that adding type IV collagen to the graft would be more effective than using type I collagen alone for promoting regeneration in situ. The use of a type I and type IV mixed collagen microsponge might promote cellularization and differentiation, making the procedure easier.

Previous studies demonstrated the existence of circulating endothelial and smooth muscle progenitor cells, which contribute to vasculogenesis and angiogenesis.[19-21] Recently, it was shown that adult bone marrow cells can differentiate into vascular endothelial cells and SMCs. Han and associates[22] found that bone marrow cells contribute to the formation of neointimal lesions in the injured vessels of mice. Shimizu and associates[23] and Sata and coworkers[24] demonstrated that $\alpha$-actin–positive cells in the neointimal lesions of allografts were colocalized with $\beta$-galactosidase positively stained cells in a chimeric mouse expressing $\beta$-galactosidase in its bone marrow cells. Therefore the ideal condition for vascular reconstruction would involve a PLGA-collagen patch to which circulating progenitor cells would migrate and which would support their survival in situ. The concept of regeneration with the PLGA-collagen patch without precellularization is based on the idea of supplying an in situ bioreactor and incubator for vascular progenitor cells. In further investigation, we have to evaluate the differences in the cellular ingrowth caused by the species differences and operative age.

Regarding the biodegradable scaffold, as previously reported,[25,26] the choice of biodegradable polymer is important. Several factors, such as degradation time, biocompatibility, and ease of handling for surgical intervention should be improved to make a new material preferable to a conventional one. Considering these factors, in this study we selected the PLGA mesh, which is already used as a surgical material and has a structure that is suitable for compounding with collagen microsponge. As we move toward the application of this patch to the systemic circulation, we are currently attempting to improve the scaffold design by reinforcing the outside with a woven poly-L-lactide fabric.

The major limitations of this study are the small number and short durations of the observations. The size of the implanted patch was also relatively small. Therefore the long-term findings regarding the efficacy of this material without precellularization might not be the same for a large conduit implanted into the right ventricular outflow tract or if another model is used. However, we believe that our strategy without precellularization might be a useful option for a novel tissue-engineering technique. The use of a xenogenous substance, such as collagen, might be a limitation, although prosthetic vascular grafts coated with collagen are already widely used in clinical situations. Moreover, for the clinical use of this graft in the repair of congenital heart disease, its durability and potential for growth should be evaluated over a long-term follow-up period. Nevertheless, this study is valuable as the first report of a newly developed bioengineered material that is feasible for grafting in cardiovascular surgery. Further investigation will be directed toward evaluating its potential for clinical applications.

## Conclusion

In the present study the PLGA-collagen microsponge patch showed good in situ cellularization and synthesis of extracellular matrix in a dog model for reconstruction of the pulmonary artery. The PLGA-collagen patch has promise as a bioengineered material for the reconstruction of autologous tissue in cardiovascular surgery.

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