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EVOLVING TECHNOLOGY

PATCH AUGMENTATION OF THE PULMONARY ARTERY WITH BIOABSORBABLE POLYMERS AND AUTOLOGOUS CELL SEEDING

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Objective: In recent years bioabsorbable synthetic or biologic materials have been used to augment the pulmonary artery or the right ventricular outflow tract. However, each of these polymers has one or more shortcomings. None of these patch materials has been seeded with cells. Thus, we have tested a fast-absorbing biopolymer, poly-4-hydroxybutyric acid, with autologous cell seeding for patch augmentation of the pulmonary artery in a juvenile sheep model.

Methods: Vascular cells were isolated from ovine peripheral veins ($n = 6$). Bioabsorbable porous poly-4-hydroxybutyric acid patches (porosity > 95%) were seeded on 3 consecutive days with a mixed vascular cell suspension ($21.3 \pm 1.3 \times 10^6$ cells). Forty-five (± 2) days after the vessel harvest, 1 unseeded and 6 autologously seeded control patches were implanted into the proximal pulmonary artery. The animals received no postoperative anticoagulation. Follow-up was performed with echocardiography after 1 week and before explantation after 1, 7, and 24 weeks (2 animals each) for the seeded control patches and after 20 weeks for the nonseeded control patch.

Results: All animals survived the procedure. Postoperative echocardiography of the seeded patches demonstrated a smooth surface without dilatation or stenosis. Macroscopic appearance showed a smooth internal surface with increasing tissue formation. Histology at 169 days demonstrated a near-complete resorption of the polymer and formation of organized and functional tissue. Biochemical assays revealed increasing cellular and extracellular matrix contents. The control patch showed a slight bulging, indicating a beginning dilatation.

Conclusion: This experiment showed that poly-4-hydroxybutyric acid is a feasible patch material in the pulmonary circulation. (J Thorac Cardiovasc Surg 2000;120:1158-68)

Biodegradable and absorbable material has been broadly used for reconstruction of the atrial wall,[1] pericardium,[2] and the right ventricular outflow tract.[3] In surgery for congenital cardiac disease the ideal material would be a nonthrombogenic living tissue that would allow growth. The polymer used in the above-mentioned studies, poly-3-hydroxybutyrate, had the significant potential shortcoming of a long degradation time. No studies have been reported with a bioabsorbable matrix and autologous cell seeding for patch augmentation of the pulmonary artery. In this study we tested a new, highly flexible, and rapidly degrading polymer, poly-4-hydroxybutyrate (P-4HB), seeded with autologous vascular cells to augment the main pulmonary artery in an ovine in vivo model.

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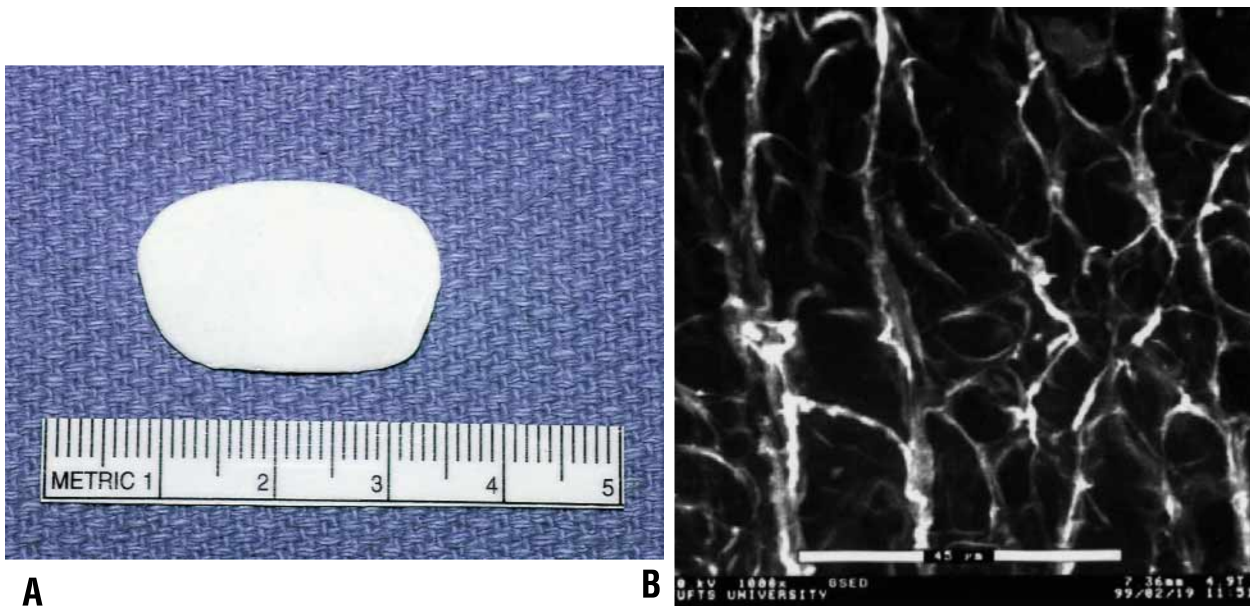

Fig 1. A, Polymer scaffold (P-4HB) 10 × 25 mm with a thickness of 1.6 mm. **B,** Surface electron microscopy of highly porous (porosity >95%) P-4HB (PHA4400). (Original magnification 500×.)

Materials and methods

The general approach to cell isolation, culture, sorting, and seeding was described in detail previously.[4] Several modifications have been made in this study.

Cell isolation and culture. Segments of peripheral limb vein (2-3 cm) were harvested from 6-week-old Dover lambs (n = 6). The vessels were minced into 1-mm^2 pieces and cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco BRL; Life Technologies Inc, Rockville, Md) supplemented with 10% fetal bovine serum (Sigma Chemical Company, St Louis, Mo), 1% L-glutamine, penicillin, and streptomycin (Sigma) on P100 dishes (Corning Inc, Corning, NY). After migration of cells onto the dishes (7-10 days after the harvest), the cells were serially passaged on 1% collagen-precoated tissue culture ware (Corning Inc). Approximately 37 ± 2 additional days were needed to obtain 12 confluent T75 tissue culture flasks. Representative cell samples were labeled with an acetylated low-density lipoprotein (LDL) marker (Biomedical Technologies Inc, Stoughton, Mass) that is selectively taken up by endothelial cells through the scavenger pathway. After 24 hours' incubation, the cell populations were analyzed by use of a fluorescence-activated cell sorter to determine the ratio of LDL-positive (endothelial cells) and LDL-negative cells (smooth muscle or fibroblasts). The analysis revealed a ratio of 5% ± 1% LDL-positive (endothelial cells) and 85% ± 1% LDL-negative cells (smooth muscle cells or fibroblasts). The remaining 10% could not be clearly classified in one of the two populations.

Bioabsorbable polymer. The P-4HB (PHA4400) was provided by Tepha, Inc (Cambridge, Mass). The size of the patch was 10 × 25 mm, with a thickness of 1.6 mm (Fig 1, *A*). A combination of salt-leaching technique[5] and solvent evaporation[6] was used to create a highly porous material (porosity >95%, pore sizes of 180-240 µm; Fig 1, *B*). In a recent study with 3 different subcutaneously (rat) implanted PHA 4400 discs (film, 50% or 80% porosity), we evaluated an in vivo degradation time between 20 weeks for the polymer with 80% porosity and 56 weeks for the film (Stock and associates, 1999, unpublished results). The patches were sterilized with cold ethylene oxide.

Cell seeding. Before cell seeding, the patches were precoated with 0.1 mg/mL collagen type I (Vitrogen 100; Cohesion Inc, Westford, Mass) for 24 hours to improve cell-polymer attachment. After 37 ± 2 days in cell culture, mixed vascular cells (endothelial cells, smooth muscle cells, and fibroblasts; $21.3 \pm 1.3 \times 10^6$ cells each time) were dripped onto the inside of the patch (seeding time, 10 minutes) on 3 consecutive days. The patches were cultured in high-glucose Dulbecco's modified Eagle's medium (GIBCO, BRL) supplemented with 10% fetal bovine serum (Sigma), 1% L-glutamine, penicillin, and streptomycin (Sigma) in tissue culture plastic ware (Corning Inc).

After 1 final incubation day in culture media, the constructs were implanted.

Implantation. At 45 ± 2 days after the initial cell harvest, the same 6 animals from which the initial vessel was harvested (average weight, 25 ± 5 kg) underwent augmentation of the pulmonary artery with an autologous tissue-engineered patch. In one other animal an acellular patch was implanted (control patch with the same precoating and culture conditions before implantation but no cell seeding). Anesthesia was induced with a 2-mg/kg dose of ketamine, a 0.02-mg/kg

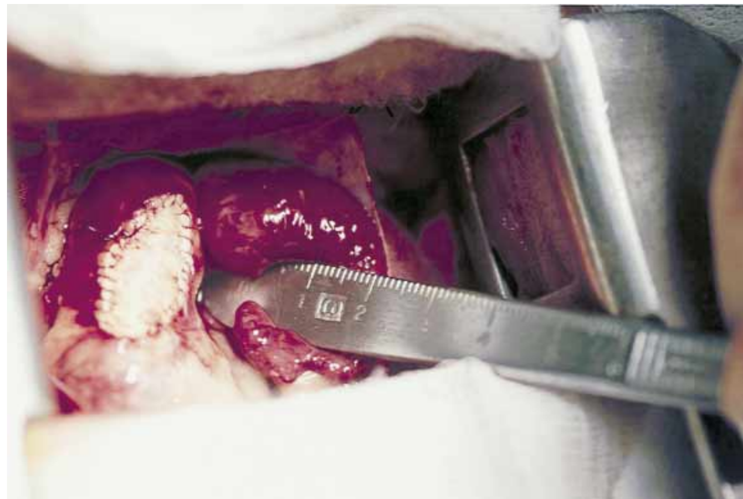

Fig 2. Implanted patch in the main pulmonary artery.

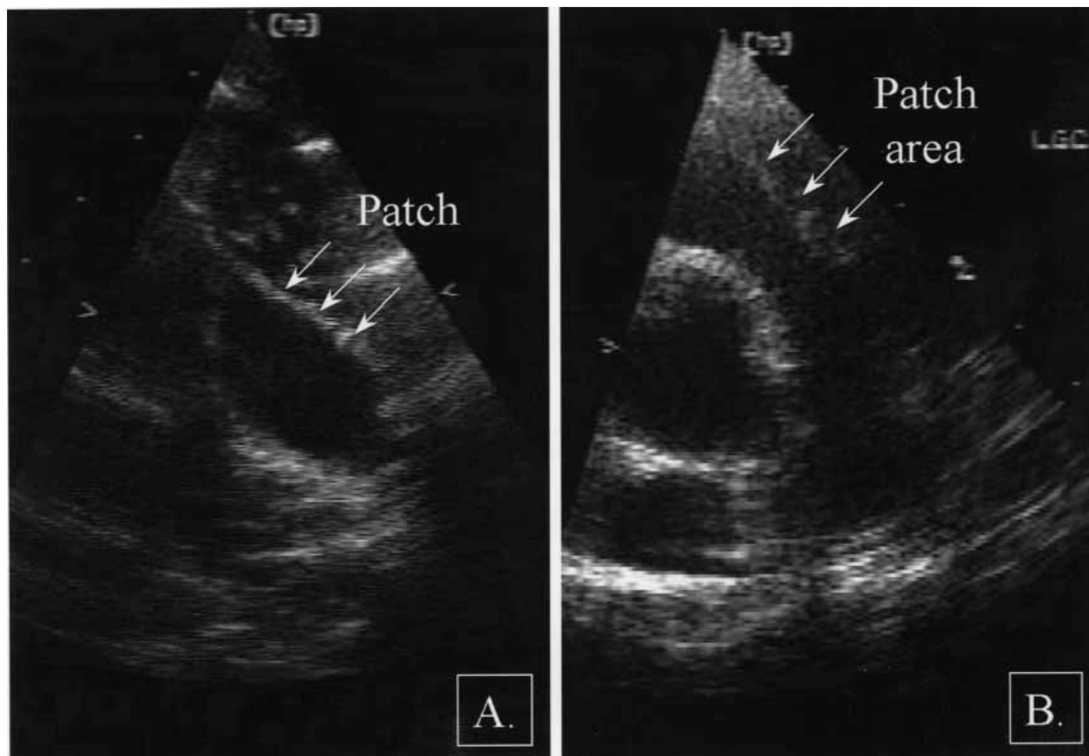

Fig 3. Echocardiographic assessment. **A,** Long axis after 1 week of implantation. Enhanced echocardiographic density of patch material. **B,** Long axis after 26 weeks of implantation. Patch area is undistinguishable from native pulmonary artery.

dose of atropine, and an intravenous bolus infusion of 2 mg/kg of propofol. Anesthesia was maintained with inhalational isoflurane. Before skin incision, an intercostal nerve block with 0.25% bupivacaine hydrochloride (INN: bupivacaine) was administered. The heart was exposed by means of a left anterolateral thoracotomy entering the chest through the fourth intercostal space. By means an atraumatic vascular Cooley-Derra clamp, the proximal pulmonary artery above the valvular level was clamped tangentially as far down as tolerated by the animal. A segment of the pulmonary artery

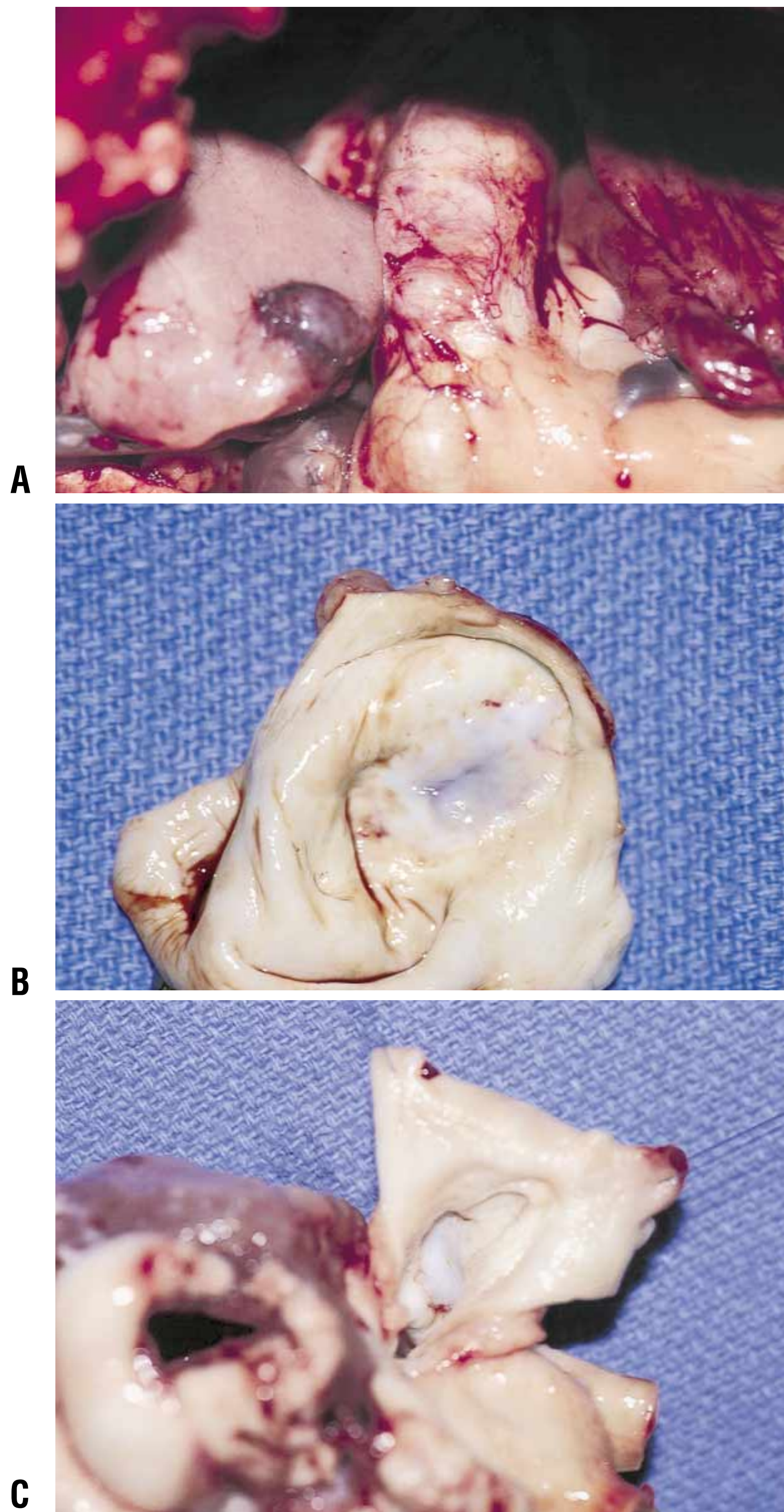

Fig 4. A, Gross appearance of seeded tissue-engineered patch 26 weeks in vivo (outside). **B,** Gross appearance of seeded tissue-engineered patch 26 weeks in vivo (inside). **C,** Gross appearance of nonseeded tissue-engineered patch 20 weeks in vivo (view into the proximal pulmonary artery).

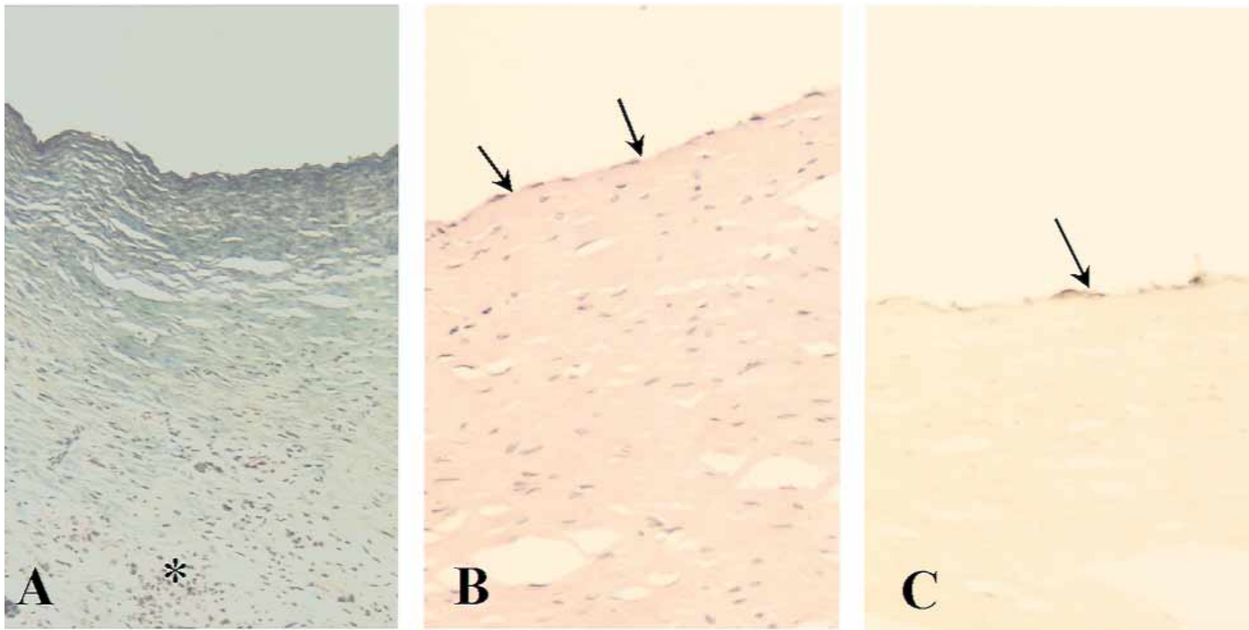

Fig 5. A, Histology of seeded patch (169 days in vivo), with stain highlighting extracellular matrix elements and showing presence of collagen *(yellow)* and proteoglycans *(green). Asterisk* indicates remaining polymer material. **B,** Seeded patch material (after 169 days in vivo) showing organized tissue formation with flattened cells on the luminal surface resembling endothelial cells *(arrows).* **C,** Immunohistochemical stain with human von Willebrand factor identifying endothelial cell lining of the luminal surface of the seeded patch. (**A,** Movat pentachrome stain, original magnification 40×; **B,** hematoxylin and eosin stain, original magnification 40×; **C,** von Willebrand factor, original magnification 100×.)

(1.5 × 3 cm) was excised, and the defect was replaced with the patch using running 5-0 polydioxanone sutures (Ethicon, Inc, Somerville, NJ; Fig 2).

The thoracic wall was closed in layers with resorbable sutures. No postoperative anticoagulation was given. All animals received 1000 mg of cefazolin (Apothecon) for the first postoperative week on a daily basis. For pain control, intramuscular buprenorphine injections were administered for the first 3 days and further on demand. 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 (National Institutes of Health publication No. 85-23, revised 1985). After 7 days in Children's Hospital research facilities, the animals were moved to an offsite indoor housing facility.

Evaluation of the engineered patches and adjacent native pulmonary artery. After implantation, Doppler echocardiography with either an Accuson 128 or Hewlett-Packard Sonos 1500 Cardiac Imager (Hewlett-Packard, Andover, Mass) equipped with a 7- to 7.5-MHz phased-arrayed transducer was used periodically to evaluate the patch area. Two-dimensional Doppler echocardiographic examination was performed on the main pulmonary artery 1 week after implantation and immediately before euthanasia after 1, 7, and 25 weeks for the seeded and 20 weeks for the unseeded patches. Evaluations included imaging of the pulmonary artery from a long- and short-axis view.

The explanted patch and the adjacent native pulmonary artery were evaluated macroscopically and histologically. Histology consisted of a hematoxylin and eosin stain for gross morphology, a Movat pentachrome stain for extracellular matrix components, and immunohistochemical staining with human von Willebrand factor for the identification of endothelial cells.

For quantification of cellular and extracellular components, histochemical assays were performed. DNA content was measured with a commercially available cell proliferation assay kit (CyQuant; Molecular Probes, Inc, Eugene, Ore) after prior proteinase K digestion.[7] For determination of collagen content, tissue 4-hydroxyproline levels were determined.[8] Elastin was quantified after tissue extraction by means of 0.1% hot oxalic acid[7] with a FASTIN elastin assay (Biocolor Ltd, Belfast, Northern Ireland), and proteoglycan-glycosaminoglycan content was quantified after tissue extraction by means of guanidine HCl[9] with a BLYSCAN proteoglycan/glycosaminoglycan assay (Biocolor Ltd). For measurement of matrix turnover and remodeling, metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) were extracted from tissue samples by use of 2 mol/L NaCl and 10 mmol/L trishydroxymethylaminomethane (TRIS).[10] MMP presence and activity were determined by a substrate gel electrophoresis

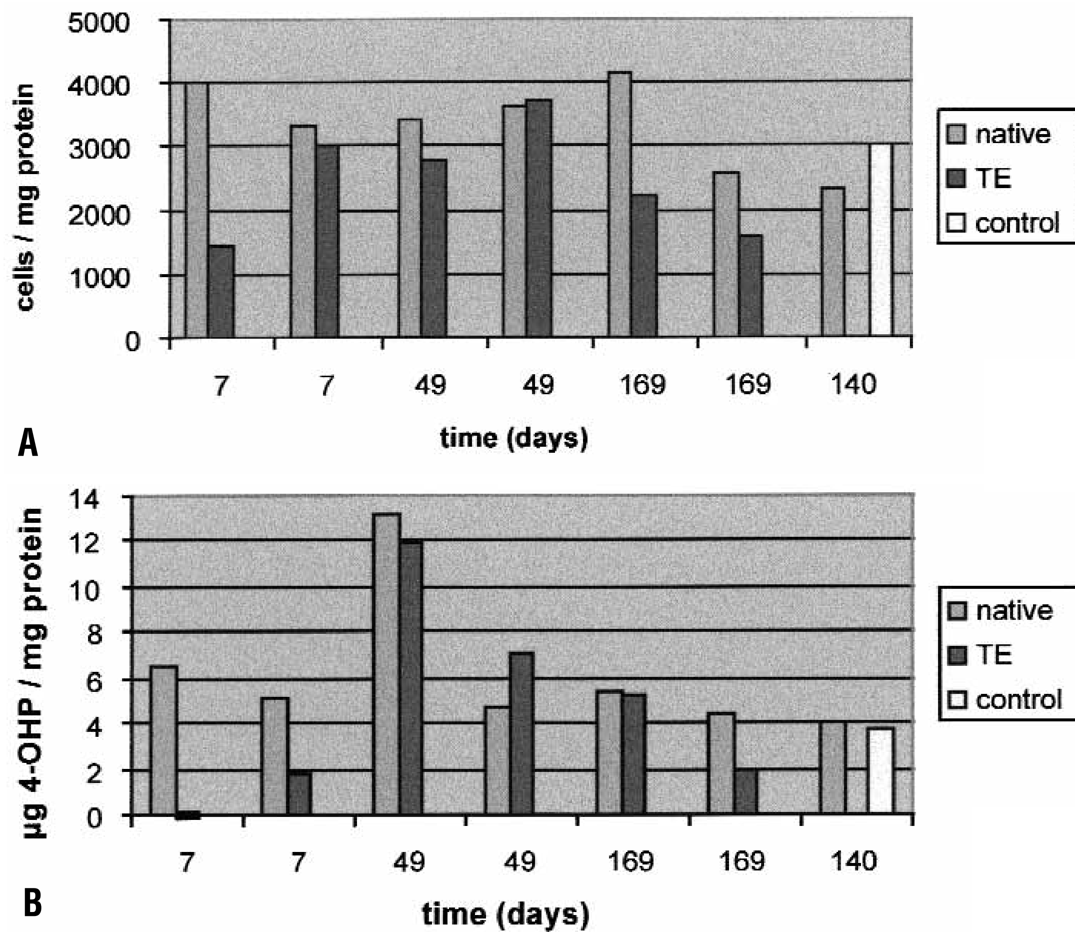

Fig 6. Content of seeded patch, unseeded patch, and native pulmonary artery. **A,** DNA; **B,** 4-hydroxyproline (*4-OHP*; collagen).

(zymography) with a sodium dodecylsulfate–polyacrylamide gel copolymerized with gelatin according to the method of Peters and associates.[11] MMP inhibitory activity (TIMP activity) was determined by the addition of bovine corneal collagenase to an appropriately diluted sample in wells containing carbon 14–radiolabeled collagen. For protein determination, protein was extracted by means of 2 mol/L NaCl and 10 mmol/L TRIS.[10] Extracted protein content was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's specifications, with bovine serum albumin as the standard.

All extracellular matrix components and active enzymes (MMPs and TIMPs) were normalized against protein concentration.

Results

All animals survived the operative procedure. During patch implantation (seeded and nonseeded patches), no bleeding occurred from the suture line, highlighting the compliance and elasticity of the polymer. Doppler echocardiography of the seeded patches revealed no signs of thrombus formation, stenosis, or dilatation. During the first echocardiographic examination (1 week after the operation), the patch material was clearly identified by a higher echocardiographic density (Fig 3, *A*). After 26 weeks, the patch became indistinguishable from the surrounding native pulmonary artery (Fig 3, *B*).

Because of technical problems during induction of anesthesia, the control animal died before the echocardiogram at 20 weeks.

Macroscopic evaluation of the seeded patches showed, after 7 weeks, an opaque, smooth, internal surface with evidence of tissue formation and neovascularization on the outside (Fig 4, *A* and *B*). The nonseeded control patch demonstrated a distinct bulging, indicating the beginning of aneurysm formation (Fig 4, *C*).

The histology at 24 weeks showed organized fibrous tissue with flattened cells resembling endothelial cells.

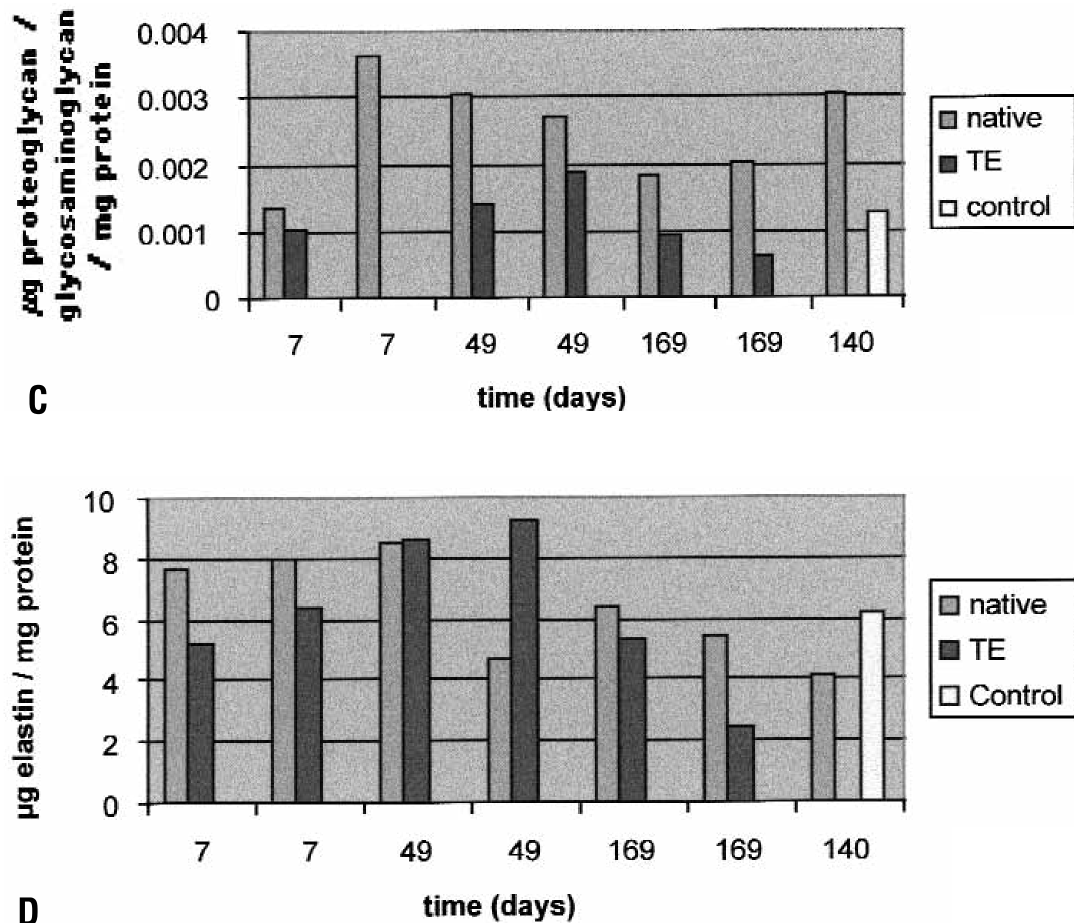

Fig 6. Cont'd. C, Proteoglycans-glycosaminoglycans; **D,** elastin. *TE,* Tissue-engineered patch.

Deep below the surface, remnants of the patch material were found surrounded by fibrous tissue. The remains of the polymer had a moderate and sharply demarcated mononuclear and focal giant cell reaction, which was exclusively limited to the polymer itself without affecting the surrounding tissue (Fig 5, *A*). A Movat-pentachrome stain revealed large amounts of proteoglycans and collagen (Fig 5, *B*). The immunohistochemical stain with human von Willebrand factor confirmed the presence of endothelial cells on the internal surface of both the seeded and unseeded patches (Fig 5, *C*).

The biochemical assays for the quantification of cellular and extracellular matrix components revealed the following results. DNA content in the seeded patch increased over the observed time to a maximum at 49 days with 91% of the native pulmonary artery, followed by a decrease toward 169 days (Fig 6, *A*). Collagen, measured as content of 4-hydroxyproline, showed a similar pattern with a maximum at 49 days (Fig 6, *B*). Proteoglycans and glycosaminoglycans expressed the same time course, with a high at 49 days and decreased levels at 169 days (Fig 6, *C*). Finally, elastin content exceeded that of the native pulmonary artery, with 150% at 49 days, but decreased toward 169 days (Fig 6, *D*). The unseeded patch evaluated at 149 days in vivo showed amounts of DNA, 4-hydroxyproline, proteoglycans-glycosaminoglycans, and elastin equivalent to that of the native pulmonary artery.

The determination of the MMPs, especially MMP-2 (Gelatinase A, the MMP that degrades basement membrane collagen) showed a maximal activity at 49 days in the seeded patches. Measurements at 169 days revealed no differences in the activity between the tissue-engineered patch and the native pulmonary artery (Fig 7). In seeded patches the levels of TIMPs reached maximal inhibitory activity at 49 days, with a decrease to undetectable levels at 169 days. The unseeded con-

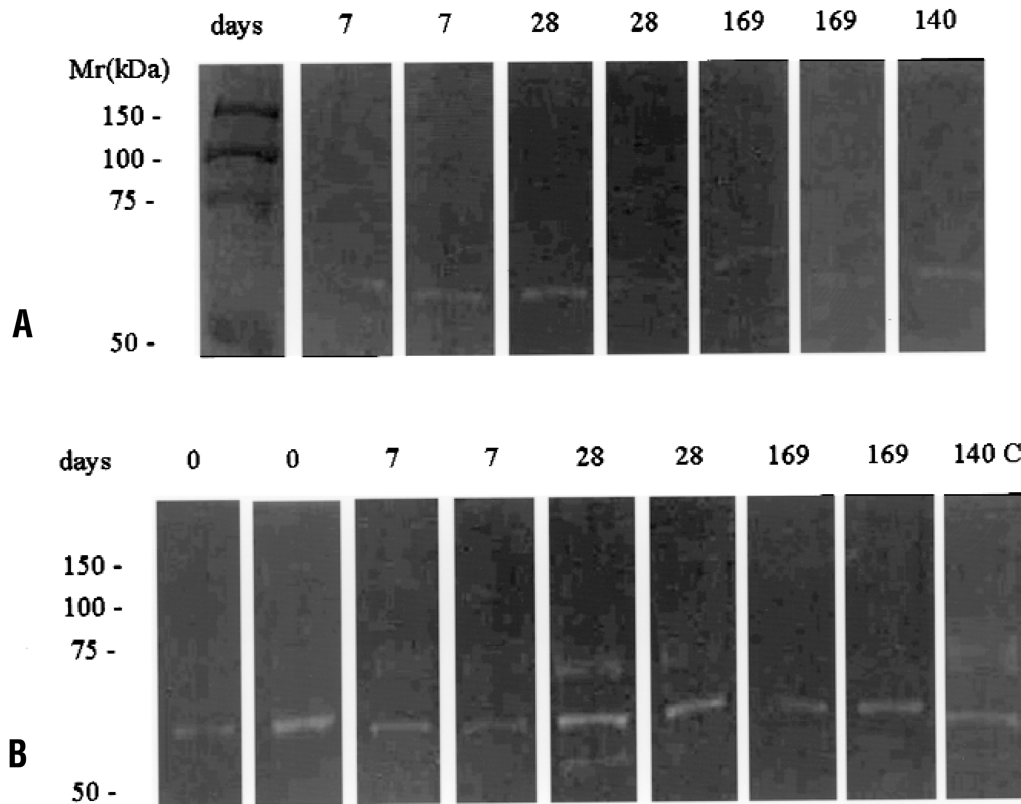

Fig 7. Substrate gel zymography of tissue samples collected from native **(A)** and tissue-engineered **(B)** pulmonary artery before implantation and at different time points up to 169 days. MMP activities are observed as zones of clearance in a polyacrylamide gel impregnated with gelatin and stained with Coomassie R-250; each band represents one animal. A band of proteolytic activity was observed at an apparent relative molecular mass *(Mr)* of 62 to 66 kd and corresponds to the active form of MMP-2. A second band at an apparent relative molecular mass of 56 to 60 kd corresponds to an autoactivated isoform of the active MMP-2. Another proteolytic band at an apparent relative molecular mass of 72 to 76 kd corresponds to the latent form of MMP-2. *C,* Control patch without cell seeding.

trol patch showed significant MMP-2 activity but no inhibitory activity of TIMPs at 140 days after implantation (Fig 8).

Discussion

Patch augmentation of the hypoplastic or stenotic right ventricular outflow tract or pulmonary artery system is a common procedure for congenital cardiac defects, such as tetralogy of Fallot. The use of nondegradable materials, such as polytetrafluoroethylene, has been associated with few complications. However, we believe that a viable vascular tissue substitute with the ability to regenerate, remodel, and grow has substantial advantages over nonvital materials. Bioabsorbable materials for the augmentation of the right ventricular outflow tract and pulmonary artery have been investigated in the past.[3] Poly-3-hydroxybutyrate was used in these studies, but it differs significantly from the P-4HB used in the present study because of a longer degradation time (>12 months to ~6 months) and a different fabrication (fiber-based configuration vs foam). In addition, it was not seeded with cells before implantation. Malm and colleagues[3] described formation of a neointima and neomedia, which was comparable with that found in the native pulmonary artery. In this study we seeded 6 patches with autologous vascular cells and implanted one further nonseeded patch into the pulmonary circulation. After 140 days in vivo, this control patch showed a bulging, potentially indicating the beginning of aneurysm formation. In contrast, Malm and associates did not observe any aneurysm formation in their study. These conflicting observations raise the question of whether seeding of biodegradable patch material is necessary, depending on the degradation time and polymer configuration.

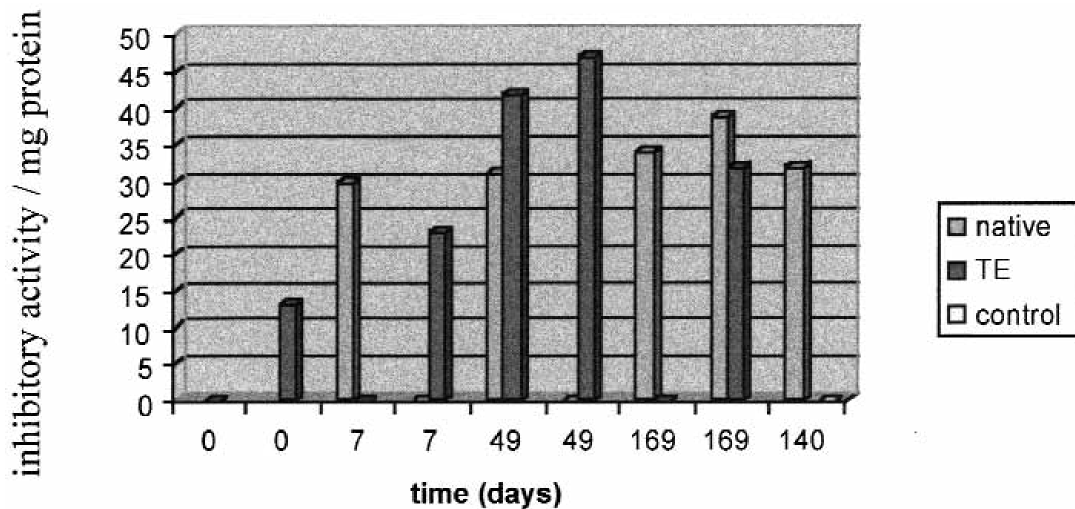

Fig 8. Activity of TIMPs.

We did observe an ingrowth of tissue from the surrounding native pulmonary artery onto the nonseeded control patch with quantitative amounts of cellular and extracellular components similar to the native pulmonary artery. Malm and associates[3] made qualitatively similar observations. Huynh and colleagues[12] recently described the concept of an acellular collagen graft (internal diameter, 4 mm) as a small-diameter vascular substitute. After implantation in a rabbit arterial bypass model (carotid artery), they observed a homogenous tissue ingrowth from both distal and proximal anastomosis and reported patency for up to 3 months. Autologous reseeding of large surfaces areas, such as a vascular prosthesis (Dacron), from the adjacent native tissue is a phenomenon seen regularly in animals but seems to occur only to a limited extent in human subjects.[13] It remains highly speculative whether the observed autologous repopulation of acellular and unseeded matrices, as observed in our studies and in those of Malm,[3] Huynh,[12] and their colleagues, can be transferred to human subjects. The unseeded patch did show quantitative amounts of cellular and extracellular components resembling the native pulmonary artery, but the bulging of the patch and the increased activity of matrix enzymes (MMPs) may indicate a disturbed remodeling process. Therefore, if some seeding becomes unavoidable, two critical questions arise. The first question concerns the source of cells, and the second question is whether the cellular components of the seeded patches are derived from the cells that were seeded onto the patch or originate from the surrounding pulmonary artery. In this study we harvested segments of peripheral limb veins corresponding to the human saphenous vein for cell isolation. In prior experiments, we used sections of carotid arteries. We believe that the use of venous segments in combination with proper cell culture techniques minimizes the risk of jeopardizing the originally supplied tissue. New research with mesenchymal stem cells[14] and circulating bone marrow–derived endothelial cells[15] may represent an additional source of cells with thus far unknown potential.

To differentiate whether the cellular components of the seeded patches are derived from the cells that were harvested and seeded onto the patch or originate from the surrounding pulmonary artery, our group has previously used fluorescent carbocyanine dyes[16] for cell labeling before seeding.[17] It was possible to identify these labeled cells for up to 6 weeks after implantation. However, successful longer term labeling and tracing without adverse effects, such as cell toxicity, remains an unsolved problem. All currently available immunofluorescent dyes are passed on during cell division to each daughter generation with equal amounts, resulting in an ongoing dye dilution. Accordingly, the longest observed follow-up of immunofluorescent dyed endothelial cells in vivo is 60 days.[18] Retroviral or adenoviral transfection of cells with a green fluorescent protein may eliminate this dilution problem and might offer an attractive pathway for long-term observations.[19,20] We are currently investigating the potential use of this technique for long-term labeling of vascular smooth muscle cells and endothelial cells.

In this study we observed an initial increase in tissue formation, with maximum deposition of cells and

extracellular matrix components and a maximum activity of tissue remodeling enzymes at 49 days. Samples from later time points showed a decrease of all components until the end point of the study after 169 days. It is unclear whether a decrease in DNA is due to cell death or greater amounts of protein in the tissue. Cell death has several potential causes. Toxicity of the polymer has been recently investigated in our group and revealed no differences among glass, Pyrex, and P-4HB (Stock and associates, 1999, unpublished results). Ischemia and cell death caused by insufficient nutritional support is one potential explanation for decreased cell count over the observed time period. The histology clearly showed ingrowth of blood vessels into the polymer, but suboptimal nutritional supply cannot be ruled out as a potential explanation for the decreased cellular content in the tissue-engineered patches. A second explanation for the cell loss is the well-known characterized mechanism of programmed cell death or apoptosis.[21,22] We are currently investigating how apoptosis may affect our tissue-engineered constructs.

Conclusion

In this feasibility study we have fabricated a viable vascular patch material that is able to function for up to 6 months in the pulmonary artery circulation. The one unseeded patch developed a significant bulging. Further work is needed to study the long-term outcome and growth ability of the patches. Advances in cell biology might offer the potential for avoidance of a vessel harvest by use of circulating pluripotential stem cells, and we are optimistic that the creation of viable tissue-engineered patches will become applicable for clinical use.

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