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PRE-CLINICAL RESEARCH

Small Vessel Replacement by Human Umbilical Arteries With Polyelectrolyte Film-Treated Arteries

In Vivo Behavior

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Objective The aim of this study was to evaluate the patency of human umbilical arteries treated with polyelectrolyte multilayers (PEMs) after rabbit implantation.

Background The development of small-caliber vascular substitutes with high patency after implantation remains a real challenge for vascular tissue engineering.

Methods Cryopreserved human umbilical arteries were enzymatically de-endothelialized and the luminal surfaces were coated with poly(styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH) multilayers. The PEM-untreated arteries and PEM-treated rabbit carotids were used as graft control. The native rabbit carotids were bypassed by grafts.

Results The Doppler ultrasound evaluation, performed in vivo, showed that all PEM-treated grafts remained patent during the full experimental period, whereas after only 1 week, no blood circulation was detected in untreated arteries. Scanning electron microscopy and histological graft examination showed pervasive thrombus formation on the luminal surface of untreated arteries after 1 week and clean luminal surface for treated arteries for at least up to 12 weeks. The arterial wall cells were identified through alpha-smooth muscle actin $\alpha\upsilon\delta$ platelet endothelial cell adhesion molecule-1 expression. The smooth muscle cells positive to alpha-smooth muscle actin were identified in adventitia and media and the endothelial cells positive to platelet endothelial cell adhesion molecule in intima. Von Kossa reaction didn't reveal any calcium salt deposits on the wall arteries, suggesting a good wall remodelling with no sign of graft rejection.

Conclusions The in vivo evaluation of human umbilical arteries treated with PSS/PAH multilayers demonstrated a high graft patency after 3 months of implantation. Such modified arteries could constitute a useful option for small vascular replacement. (J Am Coll Cardiol 2008;52:1589–97) © 2008 by the American College of Cardiology Foundation

Vascular diseases, with their high rates of morbidity and mortality in patients, are one of the most challenging research domains in medical science. Vascular grafts are of synthetic or biological origin. Synthetic grafts are most commonly made of Dacron (Invista, Inc., Charlotte, North Carolina) or polytetrafluroethylene; however, their high thrombogenic surface and poor mechanical properties limit their use as small-caliber grafts. For this reason, the use of autologous vessels (saphenous veins) constitutes an alternative choice for patients in need of small-caliber arterial reconstruction. The use of autografts is nevertheless hampered by limited availability and suitability as the result of extensive peripheral vascular diseases and/or their previous uses in bypass surgery.

An alternative vessel source could originate from allograft banks (1–3). Arterial allografts usually are used for in situ treatment of infected prosthetic grafts (4,5). A major

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Abbreviations and Acronyms

CLSM = confocal laser scanning microscopy
H&S = hematoxylin and eosin/safran
HUA = human umbilical artery
PAH = poly(allylamine hydrochloride)
PECAM = platelet endothelial cell adhesion molecule
PEM = polyelectrolyte multilayer film
PSS = poly(styrene sulfonate)
RPMI/BSA = RPMI1640 containing 0.5% bovine serum albumin
SEM = scanning electron microscopy
SMA = smooth muscle actin
SMC = smooth muscle cell
UC = umbilical cord
UnA = untreated artery

advantage related to allografts is their greater abundance compared with autografts and their adequate vascular architecture for small vessel replacements. Nevertheless, cryopreserved arteries exhibit several limits such as an increased risk of thrombogenicity due to endothelium loss (6–8) and an increased risk of intimal hyperplasia formation due to a decrease of mechanical properties (9,10). Thus, innovative technologies aimed to modify small-diameter natural grafts to obtain functional blood vessels with antithrombogenic and adequate mechanical properties comparable with native vessels constitute challenging research domains and are of the highest clinical importance. Attempts have been made to reduce the inherent thrombogenicity of vascular graft surfaces by autologous fibrin coating (11–13) or by integrating some of the processes specific to vascular endothelium function such as heparin (14–16). However, the strongest drawback of the use of heparin remains the risk of heparin-induced thrombocytopenia (17,18). Moreover, their short lifespan and the transient coating lead often to graft failures and after its crosslinking onto luminal surface of natural vessels it decreases arterial compliance (19,20).

The layer-by-layer self assembly of polyanions and polycations and the resulting polyelectrolyte multilayer films (PEMs) constitute excellent candidates for tissue engineering applications because of their biocompatibility and bioinertness. This approach also was used for blood vessel coating (21). Elbert et al. (22) found that the use of polylysine/alginate PEM prevents cell attachment. More recently, Thierry et al. (21) deposited hyaluronan/chitosan films directly on damaged arteries. Moreover, this "in vitro" experiment exhibited very weak platelet and leukocyte adhesion on films ended by a polyanion layer, thus avoiding the thrombogenic process.

We also demonstrated recently the possibility of depositing a poly(allylamine hydrochloride)/poly(styrene sulfonate) (PAH/PSS) multilayer directly inside arteries that originated from umbilical cords (UCs) (23). We showed that, after the deposition of such a film built with only 3.5 pairs of layers, the initial compliance of the tissue was recovered. Such a recovery is of main importance in particular to avoid an intimal hyperplasia at the anastomotic site (24,25).

The aim of the present work, then, was to use this strategy to coat the lumen of UC arteries by $(PAH/PSS)_3$ films with the objective of conferring them patency properties (antithrombogenic properties and good mechanical behavior). We chose the PAH/PSS system because it is known to be one of the most stable PEM systems and should be resistant to enzymatic degradations, and also because the deposition of 3.5 pairs of layers restored the initial compliance of umbilical arteries (26). We directly checked the validity of the concept in an animal model and bypassed native carotids with PEM-treated cryopreserved xenografts (human umbilical artery [HUA]). The main reason for the choice of HUA as vascular blood substitutes in these experimental models was that xenogenic materials allowed us to test a source of abundant products for human use.

Methods

Polyelectrolyte and graft arteries preparation. We purchased PSS and PAH (molecular weight = 70 kDa) from Sigma (St. Louis, Missouri) and used them without further purification. The PEMs were built from PSS and PAH solutions at 5 mg/ml dissolved in 10 mmol/l Tris and 150 mmol/l/ NaCl buffer, pH 7.4. The arteries were carefully harvested from UCs and cryopreserved as previously described (23). After 3 months of storage, the cryopreserved arteries were thawed in 37°C water and de-endothelialized by an enzymatic digestion with trypsin/ethylene diamine tetraacetic acid. Rabbit-cryopreserved carotids, used as control, were subjected to the same protocol.

Deposition of PEM into de-endothelialized allo and xenogenic arteries. The PAH solution was incubated inside the de-endothelialized arteries for 15 min. The arteries were extensively rinsed with Tris/NaCl buffer. In accordance with this previous procedure, the PSS and PAH were adsorbed alternatively for 15 min, with 2 consecutive adsorption steps being separated by 3 rinsing steps of 15 min with buffer. Thus, a $(PAH\text{-}PSS)_3$ film was progressively built. Untreated arteries (UnAs) also were subjected to several rinses with buffer and used as control. After PEM build-up, arteries were stored 12 h at 4°C in RPMI1640 medium (Sigma) supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone (Gibco BRL, Grand Island, New York).

Rabbit carotid bypass surgery. Animal care complied with the Principle of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication no. 80-23, revised 1978). A total of 18 New Zealand White rabbits (male, average weight 3 to 3.5 kg, CEGAV, Saint-Mars-d'Egrenne, France) were used for common carotid bypass surgery. Anaesthesia was induced by intravenous injection of sodium pentobarbital (40 mg/kg, Ceva Santé Animale, Libourne, France). After systemic heparinization (300 IU/kg, Sanofi Synthelabo, Paris, France), the common carotid artery was isolated and divided between vascular clamps to stop blood flow. A proximal longitudinal arteriotomy was made, and one end of grafts

(untreated [n = 6] and arteries treated with PEM [n = 12]) were anastomosed to the artery end-to-side, with continuous sutures. The distal anastomosis was performed similarly, and the native carotids were bound to shunt the blood flow through the implanted grafts. Neither anticoagulant nor antiplatelet agents were administered, except for the intraoperative heparin. After 1, 4, 7, and 12 weeks of implantation, the animals were anesthetized, grafts were removed, and the animals were sacrificed by anaesthetic lethal dose.

Doppler ultrasound examination. Post-operatively, the graft patency was evaluated, regularly up to 12 weeks, by manual palpation of the pulse and direct observation of blood flow with echo-Doppler technique (Sonos 7500 System, Philips Healthcare, Best, the Netherlands). The vessels' diameters in vivo were estimated in transversal and longitudinal axes. The technique is based on a "point-to-point" reconstruction of the vessels' inner circumference. The presence of thrombosis, stenosis, dilation, or anastomotic pseudoaneurysm formation was also checked. The native carotids were used as control.

Scanning electron microscopy (SEM) examination. The grafts were collected and washed twice with physiological serum. They were fixed and prepared as previously described (26). The vessels were observed on an Autoscan S240 Cambridge scanning electron microscope (Cambridge, United Kingdom) at 80 kV.

Histological and immunohistochemistry examination of grafts. Explanted grafts were dipped in 10% buffered formalin and embedded in paraffin. Transverse sections were cut at 4 different levels, mounted on glass slides, and subsequently stained with hematoxylin and eosin/safran (H&S) and van Gieson. Mineralization of the treated arteries after 12 weeks of implantation was estimated by the use of the von Kossa reaction according to the methods of Manji et al. (27). The H&S-, van Gieson-, and von Kossa-stained areas were viewed by light microscopy (Olympus IX 50, Center Valley, Pennsylvania). Immunohistochemical studies were performed on deparaffinized sections after epitope restoration, with antibodies directed against α-smooth muscle actin (SMA) (1:200, Dako,

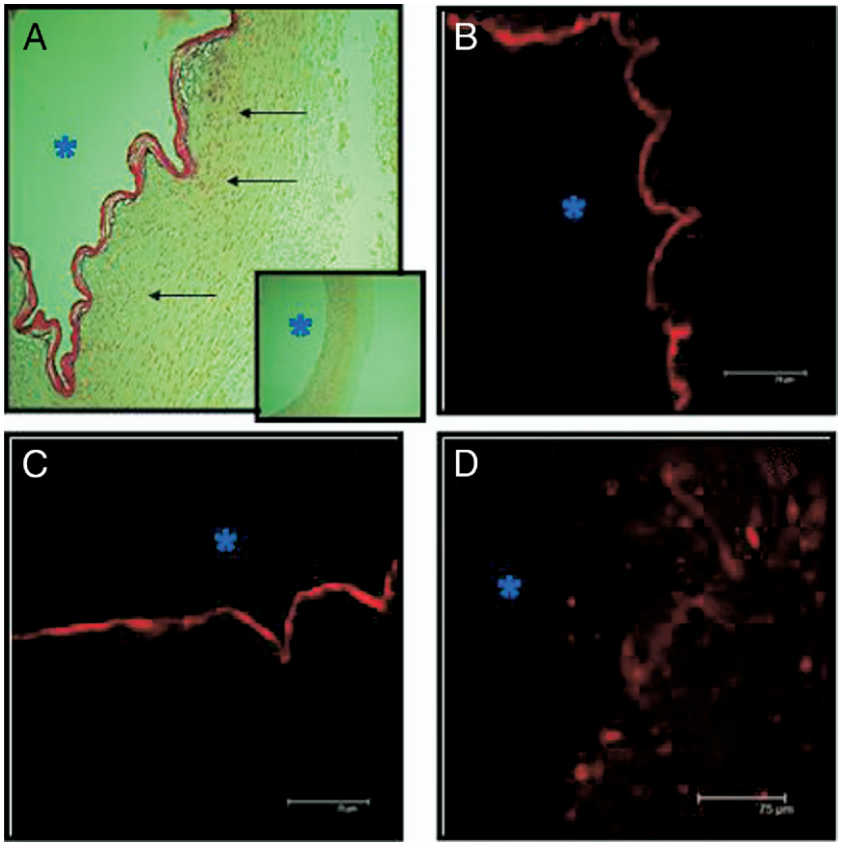

Figure 1 **Evolution of PEM During the Entire Experimental Period**

(A) Hematoxylin and eosin/safran histological investigation of (poly(allylamine hydrochloride)/poly(styrene sulfonate))$_3$-treated arteries and untreated arteries before implantation (magnification ×25). **Arrows** show the presence of nuclei in arterial walls. Observation with confocal laser scanning microscopy of the transversal artery section treated with PEM^{rho} before implantation **(B)**, at 1 week after **(C)**, and at 12 weeks **(D)** after implantation (magnification ×40, numerical aperture 0.8, scale bars = 75 μm). **Blue asterisks** indicate the lumen localization. PEM = polyelectrolyte multilayer film.

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Table 1 Blood Velocity and Vessel Diameter Values for the Native and PEM-Treated UnA After 2, 4, 7, and 10 Weeks of Implantation

	2 Weeks		4 Weeks		7 Weeks		10 Weeks	
	Substitute	Native Carotid	Substitute	Native Carotid	Substitute	Native Carotid	Substitute	Native Carotid
Blood flow, cm/s	43 ± 11	49 ± 9	42 ± 15	54 ± 10	49 ± 11	56 ± 7	59 ± 16	65 ± 10
Diameter, cm	0.27 ± 0.05	0.25 ± 0.02	0.26 ± 0.08	0.24 ± 0.03	0.29 ± 0.07	0.25 ± 0.04	0.32 ± 0.06	0.27 ± 0.02

n = 6; values are expressed as mean ± SD.
PEM = polyelectrolyte multilayer film; UnA = untreated artery.

Glostrup, Denmark) by the use of the streptavidin/biotin immunoperoxidase method.

Confocal microscopy. Samples were incubated at 37°C for 40 min with 500 μl of monoclonal anti–PECAM-1 (1:50, Dako) in RPMI1640 containing 0.5% bovine serum albumin (RPMI1640/BSA). Subsequently, the artery segments were washed twice with 1 ml of RPMI1640/BSA to remove the excess of antibodies. These arteries were incubated for 30 min at 37°C with 500 μl of polyclonal goat anti-mouse conjugated with Alexa 488 (1:100, Invitrogen, Carlsbad, California). After 3 washes in RPMI1640/BSA, the arteries were fixed by incubation with 1% paraformaldehyde at 37°C for 20 min followed by 2 washes with RPMI1640/BSA. The transversal sections of arteries were visualized by confocal laser scanning microscopy (CLSM, SP2-AOBS, Leica, Heidelberg, Germany).

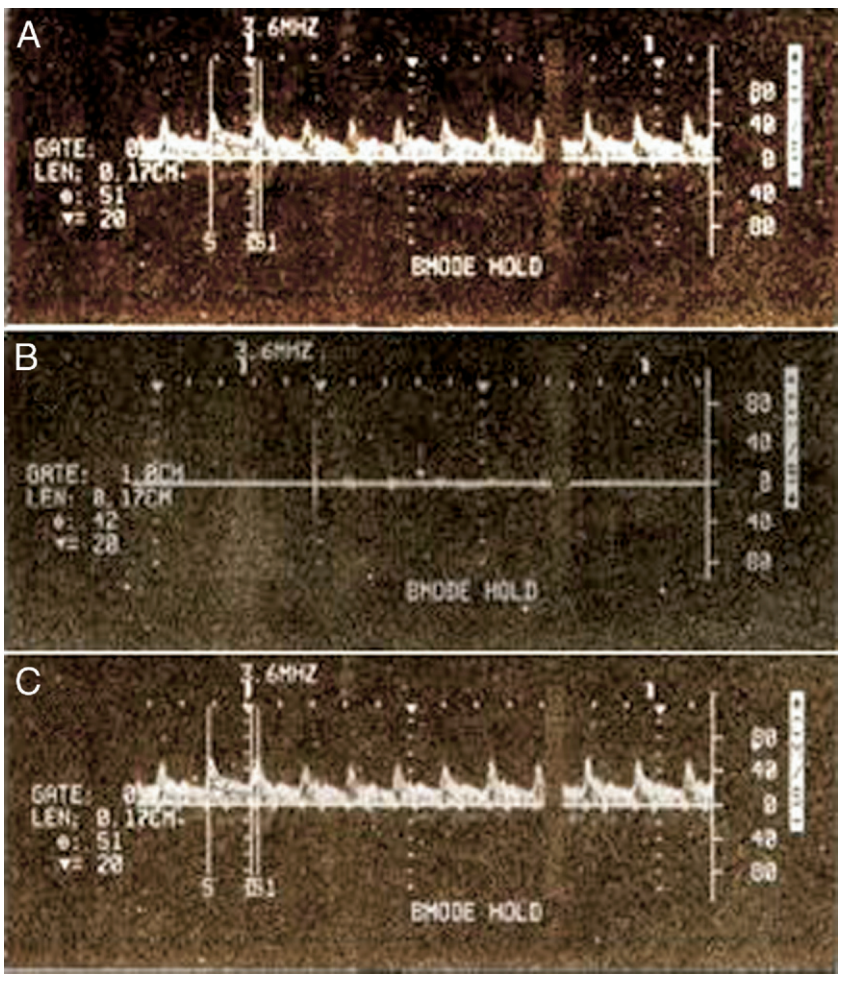

Figure 2 Blood Velocity Profile and 2-Dimensional Echo-Doppler Graft Examination

(A) Native rabbit carotid, **(B)** untreated artery, and **(C)** polyelectrolyte multilayer film-treated human umbilical artery after 10 weeks of implantation. See Online Videos 1 and 2.

Results and Discussion

The choice of rabbit as animal model, the bypass procedure, the extensive arterial injury (arteriotomy and anastomosis), and the use of a single heparin dose during surgical procedure (lifespan approximately 90 min) (28) constitute parameters that strongly increase thrombogenic risks. However, the carotid bypass is a simple process that allowed us to avoid surgical complications and trauma compared with other implantation sites such as the aorta. Furthermore, the use of nonimmunosuppressive rabbits, which increase the risk of graft rejection, represented another challenge for xenograft evaluations. All together, we thus designed conditions that increased the failure probability but that were closer to real surgical situations.

The cryopreserved HUA were de-endothelialized, and $(PAH/PSS)_3$ films were deposited into their lumen surface and implanted in rabbit carotids (bypass) for time periods of 1 week to 3 months. The UnA constituted the negative control, whereas PEM-treated rabbit carotids allowed us to compare the behavior of a modified a xenograft and an allograft.

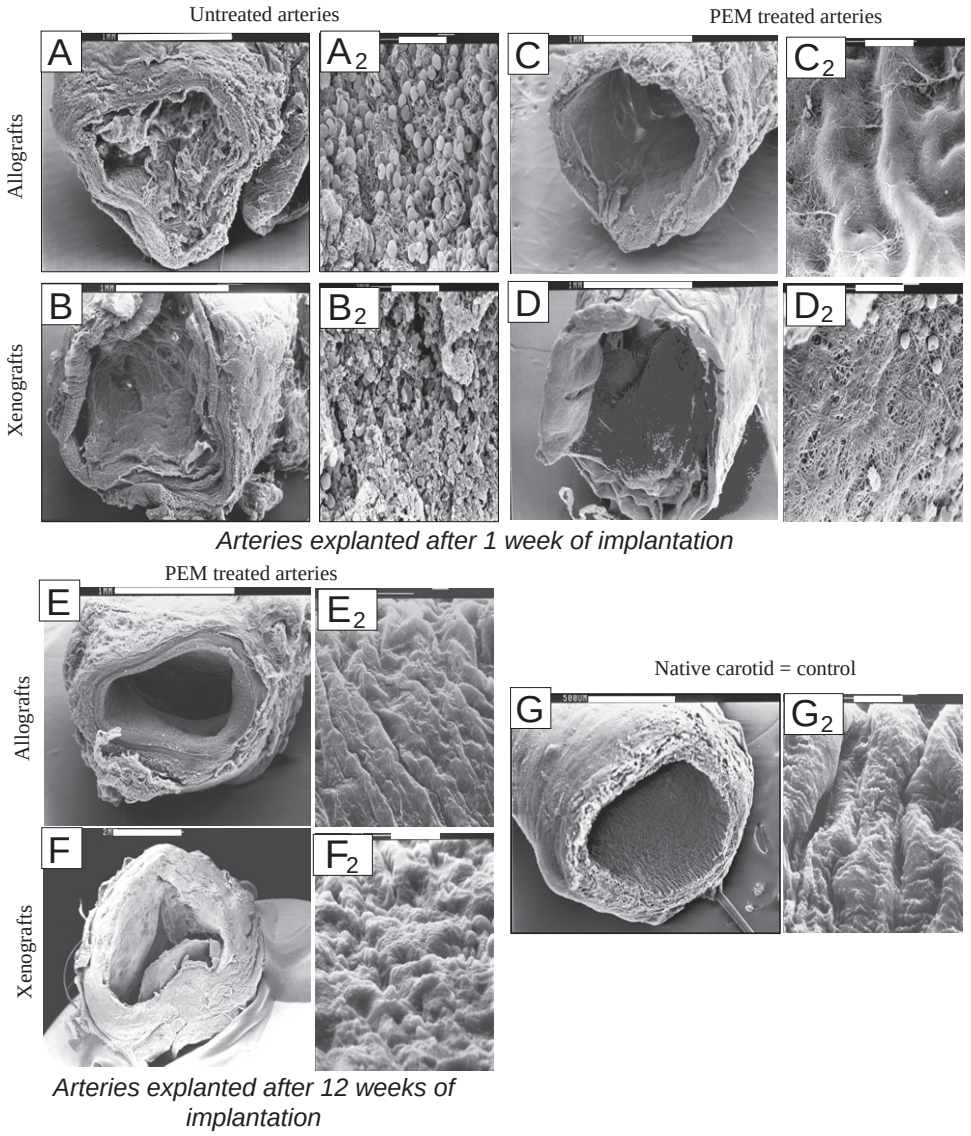

Figure 3 **SEM Images of Explanted Grafts at 1 and 12 Weeks After Implantation**

Untreated artery (UnA) **(A and B)**, with polyelectrode multilayer film (PEM)-treated **(C to F)** explanted arteries and fresh carotids **(G)**. UnA (Carotid: **A**; human umbilical artery [HUA]: **B**) occluded and with pervasive thrombus **(A_2 and B_2)** after 1 week of implantation. PEM-treated arteries (Carotid: **C and E**; HUA: **D and F**) were patent until 12 weeks **(C_2 to F_2)**. After 1 week, the internal surface of the treated arteries **(C_2 and D_2)** showed no adherent cells and platelets. After 12 weeks, the treated internal artery surfaces **(E_2 and F_2)** showed a similar morphology to the native carotid internal surface **(G_2)**. Original magnification ×400 **(A, B, C, D, E, F, G)** and ×1,000 **(A_2, B_2, C_2, D_2, E_2, F_2, G_2)**. SEM = scanning electron microscopy.

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The initial regular $(PAH/PSS)_3$ surface coating of the arteries was checked by 2 complementary approaches. First, the histological H&S staining showed a specific PEM coloration (red) (Fig. 1A) compared with UnA (Fig. 1A, insert). Moreover, we observed the presence of nuclei in the vascular wall corresponding to vascular cells (black arrows in Fig. 1A). Second, the use of a rhodamine-labeled PAH layer as the third layer with a $(PAH/PSS)_2$-PAH^{rho}-PSS surface coating showed a uniform PEM deposit and coverage of the internal surface (Fig. 1B) once observed by CLSM.

After the surgical procedure, the pulsatile carotid blood flow re-establishment was visually observed after clamping removal. All animals (n = 18) survived during the subsequent post-operative investigation period (until 3 months). No apparent neurological, hemorrhagic, or infectious complications were observed in any of the animals during the explored experimental time. The in vivo graft patency was evaluated with a noninvasive technique consisting of an echo Doppler examination performed regularly, every 2 weeks between weeks 2 and 10 (Table 1, Fig. 2). For PEM-treated arteries, the blood flow measurement revealed good blood vessel permeability during the entire period of investigations without evidence of aneurysm formation (Online Video 1). For UnA, no Doppler signal was detected after only 2 weeks of implantation (Online Video 2), reflecting a failure blood circulation.

We explanted the grafts after 1, 4, 7, and 12 weeks of implantation and submitted them to macroscopic, SEM, CLSM, and histological examinations. We will mainly focus on the most important data from the artery harvestings performed at 1 and 12 weeks. For all implantation times, no tissue disintegration and graft rupture at the anastomosis sites were observed, which constitutes a signature of excellent graft behavior and an absence of size mismatch between graft and native carotid (4).

Previous observations were confirmed by SEM examinations of explanted grafts, which showed an occluded lumen for UnA (Figs. 3A and 3B), with an internal wall clearly showing a pervasive thrombus for UnA (Figs. $3A_2$ and $3B_2$). The thrombus formation also was confirmed after examination of the histological sections of arteries stained by H&S (Figs. 4A and 4B).

The PEM-treated arteries showed that all grafts (xeno/allografts) were patent after 1 week (Figs. 3C and 3D) and

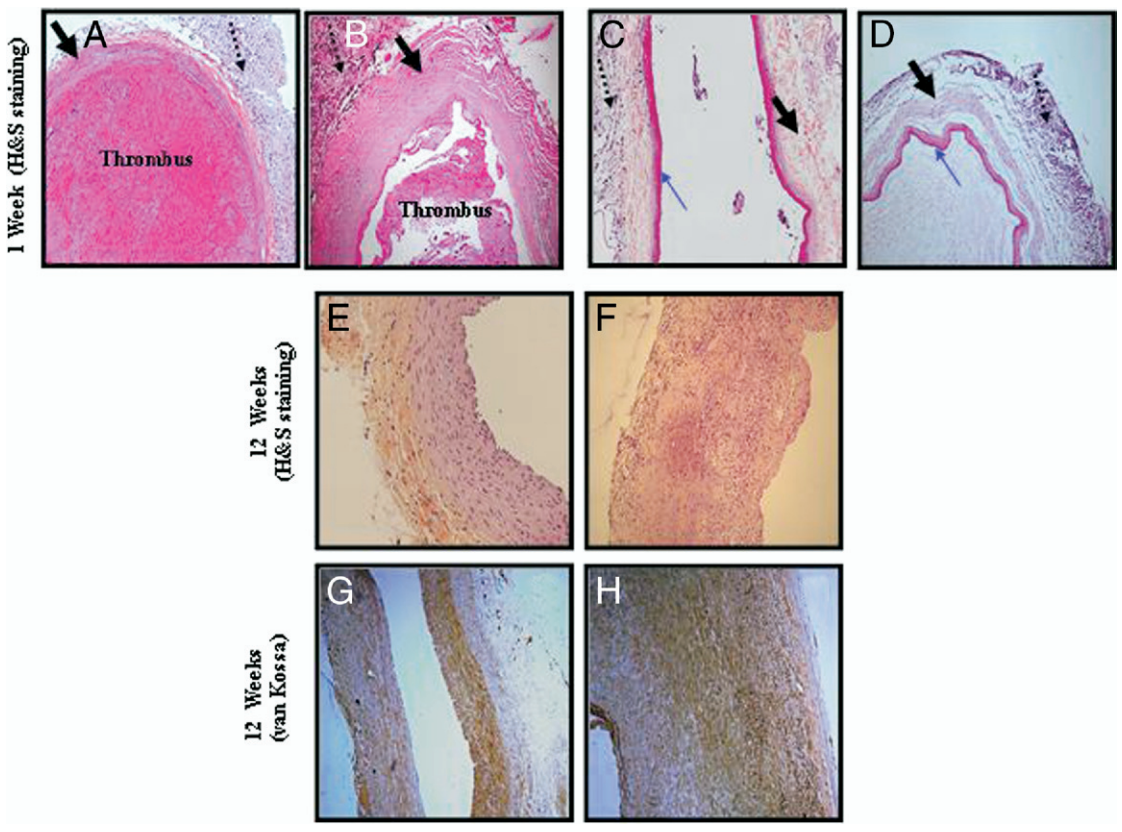

Figure 4 Histological Examinations of Explanted Grafts at 1 and 12 Weeks After Implantation

UnA (Carotid: **A**, HUA: **B**), with PEM-treated arteries after 1 week (Carotid: **C**, HUA: **D**) or 12 weeks (Carotid: **E**, HUA: **F**). **Dotted arrows** indicate inflammatory reactions at adventitia after 1 week. **Black arrows** indicate the absence of vascular cell, and **blue arrows** indicate red PEM after 1 week of implantation. The von Kossa reaction **(G and H)** at 12 weeks performed in the explanted arteries treated with PEM indicate the absence of wall calcification. Magnification ×40. Abbreviations as in Figures 1 and 3.

up to 12 weeks of implantation (Figs. 3E and 3F). The SEM internal surface observations for the PEM-treated vascular wall did not show any adhering blood cells after 1 week (Figs. $3C_2$ and $3D_2$) with a fully comparable aspect described before surgery (26). After 12 weeks of implantation, the lumen of PEM-treated arteries appeared covered by cells (Figs. $3E_2$ and $3F_2$). Compared with fresh carotid (Fig. $3G_2$), the SEM morphological examination of PEM treated arteries showed an alike surface aspect. This could be a result of endothelial cell (EC) colonization (see the following text).

The explanted treated arteries were also subjected to H&S and von Kossa histological examinations. The images also demonstrated a perfect blood permeability of luminal surface arteries treated by PEM for all explored time periods (Figs. 4C to 4E). After 1 week of implantation, the wall structure examinations showed (Figs. 4C and 4D): 1) the presence of the expected chronic inflammatory cells in arterial adventitia (Figs. 4C and 4D, dotted arrows) probably due to the healing process (29); 2) the disappearance of the cells from the media (Figs. 4C and 4D, black arrows) due to the absence of vasa vasorum, leading to apoptosis or necrosis of cells (30,31); and 3) the presence of PEM and its regular coating on the internal surface, visualized by H&S (Figs. 4C and 4D, blue arrows) and confirmed by CLSM observations (Fig. 1C), was fully comparable with the observations made before implantation (Fig. 1A).

After 12 weeks, the histological analysis of treated arteries showed (Figs. 4E and 4F): 1) the total absence of the inflammatory reaction on adventitia; 2) the presence of cells in the media wall; and moreover; and 3) an absence of wall arteries biomineralization, which is the signature for the absence of an acute graft rejection and failure strengthened by a negative von Kossa reaction (Figs. 4G and 4H) (27). All of these observations seem to confirm a healing process.

The red PEM staining was not further observed on histological arteries section after 12 weeks of implantation (Figs. 4E, 4F, and 5A). The harvested PEM^{rho} arteries after 12 weeks of implantation were observed by the use of CLSM and showed the presence of rhodamine aggregates inside the arterial wall (Figs. 1D and 5B). These observations indicate a strong PEM disturbance probably due to vascular wall remodeling. This disturbance could eventually also indicate growth of new tissue between the film layers themselves but more probably the diffusion of fragmented film parts within the wall. On the one hand, it is effectively known that PAH/PSS layers are stiff materials, favoring cell adhesion and growth, but on the other hand, the brittleness

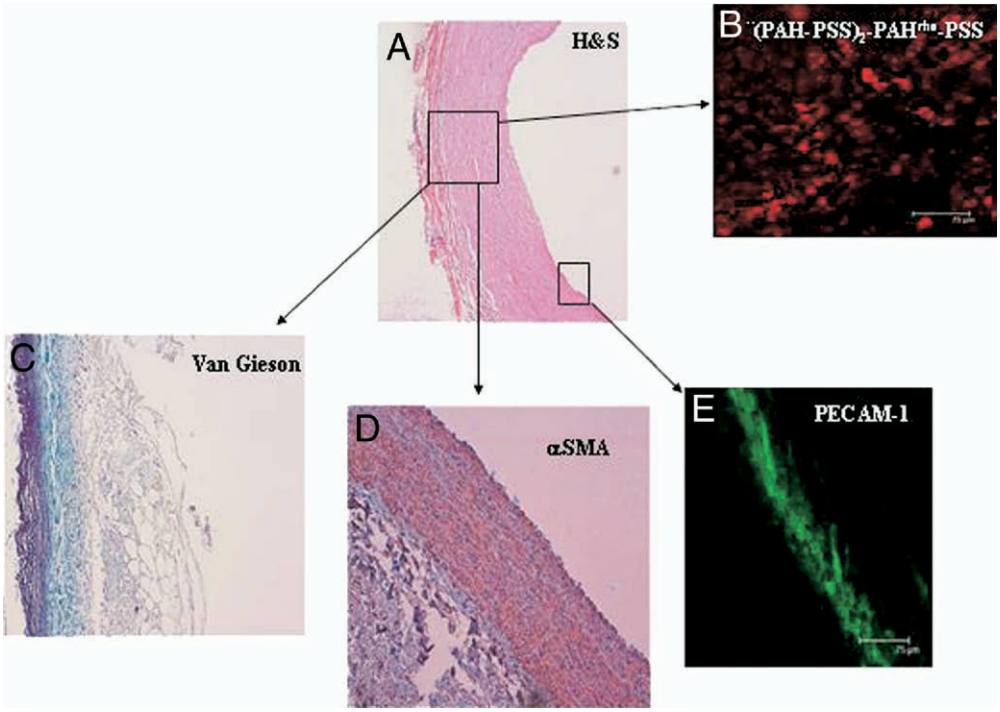

Figure 5 **Vascular Wall Remodeling, Cell Identification, and PEM Visualization After 12 Weeks of Implantation**

(A) H&S histological examination of PEM-treated arteries. **(B)** Observations with confocal laser scanning microscopy (CLSM) showed the presence of PAH^{rho} on the arterial wall surface. **(C)** van Gieson staining showed the presence of elastin fibers in media **(purple)** and collagen in adventitia **(blue)**. **(D)** Immunohistochemistry demonstrated the presence of α-SMA^{+} cells on the arterial wall (magnification ×20). **(E)** The CLSM observations showed the presence of PECAM-1^{+} cells on internal arteries surface (magnification ×40, NA 0.8, scale bars = 75 μm). H&S = hematoxylin and eosin/safran; PAH = poly(allylamine hydrochloride); PECAM = platelet endothelial cell adhesion molecule; PEM = polyelectrolyte multilayer film; PSS = poly(styrene sulfonate); SMA = smooth muscle actin.

of such films could favor the diffusion of complexed polyelectrolyte aggregates within the cell wall (32–34).

A last origin of failure of vascular grafts concerns the neointimal hyperplasia and in anastomosis sites, partly originating from an excess of smooth muscle cell (SMC) proliferation and high extracellular matrix synthesis. The present data showed a complete media regeneration. Moreover, the van Gieson staining showed vascular extracellular matrix organization with media elastin and adventitia collagen localization (Fig. 5C), with a minimal intimal hyperplasia at the anastomosis site. The media cell identification, through α-SMA expression, demonstrated that cells present in the arterial wall were α-SMA$^+$ (Fig. 5D), which is a specific marker of a SMC phenotype. The mechanism involved in the regeneration of the media vascular grafts remains unanswered. It is generally observed that the SMC recolonization constitutes the ultimate step of a series of complex interactions of both cellular and enzymatic systems, which are influenced by multiple factors (35–37). Furthermore, the vessel occlusions at the anastomosis sites due to the high SMC proliferation were described previously (38–40). Two main reasons were advanced: 1) a compliance mismatch between vascular grafts and arteries recipient; and 2) a lack of EC. Moreover, the histological analysis at the anastomosis sites showed a minimal SMC proliferation at the distal anastomosis site.

The internal surface examinations (Figs. 3E, 4F, and 5D) indicated the presence of α-SMA$^-$ cells. The identification of the cell type present on the intimal layer was visualized with the use of PECAM-1 expression on CLSM. These observations showed luminal surface of treated arteries covered with PECAM-1$^+$ cells, a signature for endothelium regeneration (Fig. 5E). The supplementary investigations, performed after 4 and 7 weeks, demonstrated the presence of EC only in the neighborhood of anastomosis sites, suggesting strongly the interstitial invasion of EC from the cut edge of the adjacent artery.

The re-endothelialization and interstitial invasion may depend on the regeneration capacity of the individual recipient and therefore be dependent on age and health status (41). The regenerative abilities of elderly animals seem to be lower than for juvenile ones in the sheep model (42). This parameter could in particular influence the migrative and proliferative function of cells and their capacity for matrix recellularization. In this respect, the use of juvenile rabbits could represent a certain limitation. Also, long-term studies are necessary to validate the benefits of the presented method as compared to other alternative approaches.

Conclusions

To our knowledge, this study represents the first report evaluating, in vivo, 1) the potential development of HUA as vascular grafts; and 2) the thrombogenicity of PEM-treated arteries. The use of HUA banks represents a bioavailable source with adapted size and with excellent handling characteristics for the surgeons. Our results demonstrate the good behavior (no sign of tissue rupture and rejection) and tissue integration of HUA.

The PAH/PSS multilayer film deposit in the luminal surface avoids the early thrombus formation because this coating approach limits the accessibility of wall collagen (which is highly thrombogenic) to thrombus factors. Vascular wall regeneration with the recolonization of SMC and EC grafts were obtained after 3 months of implantation. Such small animal models (i.e., rabbit) are inexpensive and provide a good initial in vivo environment to examine potential therapies. However, to determine whether a therapy will be effective in patients, it should be evaluated in a large animal model before clinical trials are initiated.

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Key Words: vascular tissue engineering ■ polyelectrolyte multilayer film ■ human umbilical arteries.

APPENDIX

For accompanying videos and legends, please see the online version of this article.