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Prelining Autogenic Endothelial Cells in Allogeneic Vessels Inhibits Thrombosis and Intimal Hyperplasia: An Efficacy Study in Dogs

Xuehu Wang, M.S., Yu Zhao, M.S.,[1] Zhongxue Fu, Ph.D., Yang He, M.S., Dongzhou Xiang, M.S., and Li Zhang, M.S.

Department of Vascular Surgery, First Affiliated Hospital of Chongqing Medical University, Chongqing, China

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Background. **The long-term patency rates in vascular transplants (diameter < 3.0–4.0mm) are very low due to thrombus formation and intimal hyperplasia. A possible mechanism is the loss of the endothelial cells (ECs) lining. Previous attempts to reseed ECs had poor results due to seeded cell loss, severe antigenicity, and low compliance. The objectives of this study were to generate an allogeneic vascular substitution with autogenic ECs and low antigenicity.**

Methods. **ECs from mongrels were obtained and multiplied *in vitro*, then seeded to the allogeneic vein luminal surface, which was preserved by freeze-drying radiation. The cultivated cells' secretory function was confirmed by von Willebrand factor detection. The allogeneic vascular was then transplanted into animals' necks *in situ*. The physical properties, EC state, and vascular structure of the allogeneic vascular grafts were studied.**

Results. **The secretory function of ECs did not vary *in vitro*. The expression level of MHC-II antigen in freeze-dried radiation-treated vasculature was lower than normal fresh vasculature ($P < 0.05$). ECs covered the vascular inner surface and adhered tightly after implantation. As assessed by scanning electron micrograph, most ECs adhered tightly, and the cell polarity changed in accordance with the direction of the force. Allograft blood vessels with autogenic ECs implanted showed significant decreases in both thrombosis and intimal hyperplasia.**

Conclusion. **Allograft blood vessels seeded with autogenic ECs improved the patency of small-diameter grafts in a canine model. Our study showed a significant decrease in both thrombosis and intimal hyperplasia.**

Key Words: **autogenic endothelial cells; seeding; endothelium; inhibition of intimal hyperplasia; allogeneic vascular substitute.**

INTRODUCTION

Vascular reconstruction is one type of vascular surgery operation. Currently, the long-term patency of vascular reconstruction is generally poor, with high failure rates [1, 2], especially in small-diameter vascular grafts, defined as those with an internal lumen less than 6 mm in diameter. These small-diameter grafts have low patency rates in humans due to thrombosis and intimal hyperplasia. In the first 30 d, vascular graft failure due to acute thrombosis accounts for approximately 80% of graft failures [3]. The only known completely nonthrombogenic material is endothelium; any other material that contacts the bloodstream is disposed to thrombosis. Long-term failure is due to anastomotic and intimal hyperplasia [4]. The precise reasons for anastomotic and intimal hyperplasia are still not completely understood; however, EC and smooth muscle cell dysfunctions are likely involved. Perivascular endothelial implants have been shown to be effective at decreasing intimal thickening after angioplasty of porcine carotid arteries [5, 6]. The endothelium is critical for preserving vascular homeostasis, serving not only as a physical barrier but also as a source of biologically active compounds [1]. Research has shown that xenogeneic and allogeneic endothelial implants reduce intimal hyperplasia and thrombotic occlusion [1, 5, 7].

Many vascular substitutes are currently in use. Autologous vessels seem to be the most effective for

[1] To whom correspondence and reprint requests should be addressed at, Department of Vascular Surgery, First Affiliated Hospital of Chongqing Medical University, No. 1, Youyi Road, Yuanjiagang, Yuzhong District, Chongqing 400016, China. E-mail: chongyi2007bo@gmail.com.

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angioplasty, but the source restricts these vessels in diameter and length. The use of xenogeneic vasculature is limited due to its strong antigenicity. In recent years, vascular prostheses have only been used in large vessels, but they are prone to frequent thrombosis and infection due to their low compliance and poor histocompatibility. The only difference between allogeneic and autologous vessels is the antigenicity of the former. Many methods are available to reduce the antigenicity of allogeneic vascular substitutes, including endothelium denudation, cryogenic preservation, and deep-frozen dry irradiation [8].

Due to the limited success of clinical trials, endothelial cell seeding has been largely experimented [1]. As early as 1978, EC seeding in polymeric vascular grafts has been used to improve the patency of small-diameter vessels. We obtained auto-ECs by digesting endothelium and amplified them in *in vitro* culture; they were then implanted into freeze-dried irradiated allogeneic vessels, and transplantation was performed when ECs grew solidly. We hypothesized that seeding allogeneic vessels with auto-ECs would decrease the thrombotic occlusion and intimal hyperplasia rates in mongrels. To directly test this hypothesis, we investigated the vascular changes after allogeneic vascular grafting in which auto-ECs were implanted. The information provided could benefit not only clinical therapies, but might also be important for effective maintenance and control of intimal hyperplasia over the long term. We report that the application of auto-EC implants in allogeneic vasculature resulted in no apparent toxicity, and significantly reduced thrombotic occlusion and intimal hyperplasia.

MATERIALS AND METHODS

Experimental Animal Groups and Preparation of ECs

Thirty-two adult male mongrels weighting 9.0 ± 1.0 kg obtained from the Laboratory Animal Center of Chongqing Medical University were randomly divided into three groups: vascular donor group (VDG), control group (CG), experimental group (EG). The animals were fed a standard diet and given free access to water; and were housed in animal facilities with a 12-hour light:dark schedule. Under anesthesia consisting of an intramuscular injection of 2.5% sodium pentobarbital (1 mL/kg), an incision was made in the hind limbs of the EG animals; two large saphenous veins were removed, rinsed with saphenous and serum-free medium (Dakewe Biotech Co. Ltd.) three times, and then injected with 3–5 mL of 0.2% collagenase type I (Sigma Corp.) and placed at 37 °C for 30 min. The intravenous fluid and rinse fluid with 10 mL of serum-free medium were combined and centrifuged for 8 min at 800 r/min. The supernatant was removed, and the cells were resuspended with EC Medium (Dakewe Biotech Co. Ltd.) containing VEGF, EGF, and FGF, at the density of $1.5–2.0 \times 10^5$/mL. Then it was incubated in poly-L-lysine culture-coated bottles. The primary cultures were incubated at 37 °C in a humidified 0.7% carbon dioxide atmosphere with changes of culture medium after 24 h and then after every third day. Cell viability was assessed during cell culture and was defined as the number of attached ECs counted after 18 h in culture. Primary ECs were passaged at a ratio of 1:3 when they had grown to confluence.

ECs and Its Biological Function Identification

Immunofluorescence was used to identify the ECs. The cells fixed by 75% alcohol were blocked endogenous peroxidase using H_2O_2 for 10 min and nonspecific antibody binding for 20 min (Vector Laboratories Ltd., Peterborough, UK). The primary antibody (Thermo Fisher Scientific, 1:100) were applied at 37 °C for 1 h, and the Immunol Fluorence Staining Kit (Beyotime, China) was used to visualize. Biological functional tests were performed before implantation. We changed the serum-free medium and continued the culture for 24 h before assaying the von Willebrand factor (vWF) level, which was used as a marker for EC function. We used a vWF ELISA kit (USCN Life Science and Technology Co.) to determine the vWF levels in the EC culture medium. We observed changes in EC morphology during the culture period and generated a cell growth curve *via* cell counting.

Treatment of Allogeneic Vascular Grafts and Implantation of ECs

Under anesthesia consisting of an intramuscular injection of 2.5% sodium pentobarbital (1 mL/kg), an incision was made in the necks of VDG dogs, and about 15.0 cm of the bilateral jugular vein was resected. Three to 5 mm of collagenase (0.2%) was injected into the veins to digest ECs, and the samples were incubated for 15 min and washed three times in saline. The digested vessels were frozen at –70 °C for 24 h, and then freeze-dried at –70 °C and 5 uHg for 6 h, followed by treatment with 2.5×10^6 Rad of radiation. Electron microscopy was used to detect the endothelium. For light microscopy detection, endothelium-denuded lyophilized irradiated vessels (dELIVs) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were also stained with mouse anti-dog MHC-II assess antigenicity, and normal fresh veins were used as positive controls according to the S-P immunohistochemical method. The expression of MHC-II was quantified by VIDAS System.

Half of the dELIVs were injected with a heparin saline solution (6750 U/100 mL), washed three times, and then injected 2.0–2.5 mL EC suspension (2.0×10^6/mL), placed in a glass tube that was closed at both ends and rotated six times per hour at 37 °C. After 6 hours, the EC suspension, which contained non-adherent ECs was collected, the numbers of cells were calculated, and the numbers of cells that adhered to the dELIVs were assessed. The dELIVs implanted with ECs were cultured for 9 d in medium until the ECs were firmly adhered.

Surgical Procedure

Anesthesia was administered and the incision was made as before. About 10.0 cm of the bilateral jugular vein was resected in EG and CG, and the excisional vein was used for compliance measurement. Heparin saline was used for anticoagulation in the operation. Allogeneic vascular grafts lined with ECs were transplanted into the necks of the EC-donor EG animals *in situ*; allogeneic vascular grafts without ECs were transplanted into the necks of CG animals *in situ*. Adventitia from both vessels was removed at the site of transection. The total length of the vascular grafts was about 10 cm. The vessels were connected using 8/0 sutures in an interrupted fashion to create an end-to-end anastomosis. After creation of the anastomosis, we separately blocked both ends of graft vessels, checked the status of vascular filling, and confirmed the blood flow through the vessels. After transplantation, the wounds were closed in layers to eliminate dead space. On wk 1, 4, 12, and 20, angiography was used to check the patency of transplanted vessels.

Morphometric and Physical Property Analysis

On postoperative wk 1, 4, 12, and 20, the allogeneic vascular grafts were resected under anesthesia consisting of 2.5% sodium pentobarbital (1 mL/kg) and perfused with PBS followed by formalin to fix the tissue. The allogeneic vascular grafts were isolated, trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and

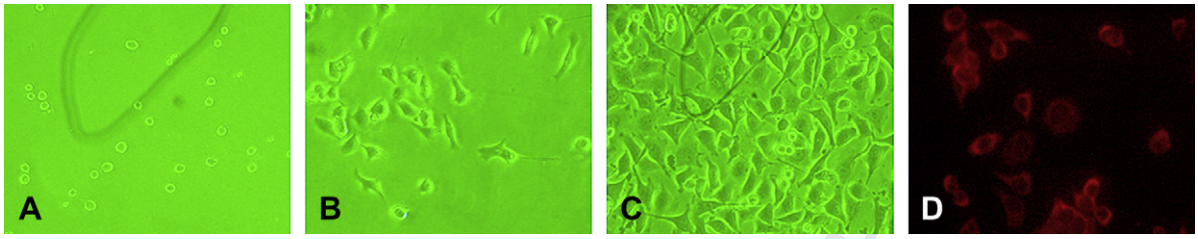

FIG. 1. Morphology of cultured ECs and Factor VIII related antigen stained. (A) Cell morphology after inoculation; (B) inoculation 24 h; (C) inoculation 7 d; (D) Factor VIII related antigen stained by immunofluorescence.

eosin. The endothelial structure was studied *via* scanning electron microscopy. Grafts were fixed in 3% glutaraldehyde for 24 h and then transferred to sodium cacodylate buffer and dehydrated in liquid CO_2, sputter-coated with gold, and observed using a scanning electron microscope. The physical properties of dELIVs were assessed using a BL-420Bio-Functional Experiment System. We measured changes in the lengths and diameters of the vasculature in response to pulling with different amounts of force. The change rate = (Stretched length – Unstretched length) /Unstretched length × 100%. Normal fresh vessels were used as positive controls. In addition, the force was also measured when the vessels of two groups were pulled off.

Statistical Analysis

All data are presented as mean ± SE. Statistical analysis was performed using a paired samples *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Cytomorphologic and Growth Kinetics and Biochemical Activity of ECs

Three hours after inoculation of primary ECs, the ECs began to adhere and looked round, after which they gradually extended to form a spindle-like shape. After training for 24 h, 80%∼90% of ECs were adherent with projections formed. The ECs progressed into the exponential phase of growth and their numbers increased substantially, and they began to become linked after 3–5 d. After training for 7 d, the cells with compacted junctions reached saturation or contacted inhibition, and began to progress into the stagnation phase. On d 7–8, the cells were passaged at a ratio of 1:3 when their numbers had increased about 7.53- ± 0.89-fold. Three days after the first passage, the cells entered the exponential phase of growth. The final number of harvested cells was $12.72 \pm 1.97 \times 10^6$, which was sufficient for implantation. The cells' growth speed accelerated as the time longer. The mark of Factor VIII related antigen by immunofluorescence showed that the ECs were stained red (Fig.1).

The results of assays of vWF levels showed that there were no differences between the original cells, the passaged cells, and the cells following transplant, indicating that the secretary function of the ECs was maintained during the *in vitro* growth process, and could exert its function after transplant (Fig. 2).

Physical Properties of Freeze-Dried Radiation-Treated Allograft B Vessels

We measured the changes between freeze-dried radiation-treated and fresh blood vessels with respect to diameter and length under different forces. No significant differences were observed horizontally or vertically ($P < 0.05$) (Fig. 3). The compliance of the freeze-dried radiation-treated blood vessels was similar to that of fresh blood vessels, and they were suitable for transplant. The greatest tension forces that the vessels could bear were different between the freeze-dried radiation-treated and fresh vessels (Fig. 4). This indicates that the freeze-dried radiation-treated vessels were stronger than normal fresh vessels in response to tension.

Antigenic Variation of Allograft Blood Vessels

The expression level of MHC-II antigen in the freeze-dried radiation-treated vessels (0.296 ± 0.01) was lower than that in normal fresh vessels (0.206 ± 0.009) ($P < 0.05$). *In vivo*, the number of inflammatory cells in freeze-dried radiation-treated vessels was also lower after transplantation (Fig. 5). This indicates that the low antigenicity could induce immune allograft rejection.

Observation of Seeding ECs

Using a scanning electron micrograph, the smooth muscle was exposed and no EC could be seen after blood

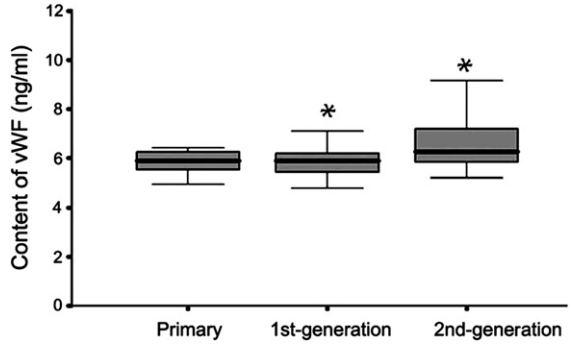

FIG. 2. The vWF contents in EC culture supernatants. The contents of vWF in primary and first and second generation ECs were, respectively, 6.63 ± 1.32 ng/mL, 5.83 ± 0.64 ng/mL, and 6.63 ± 2.51 ng/mL. * $P > 0.05$ *versus* primary ECs.

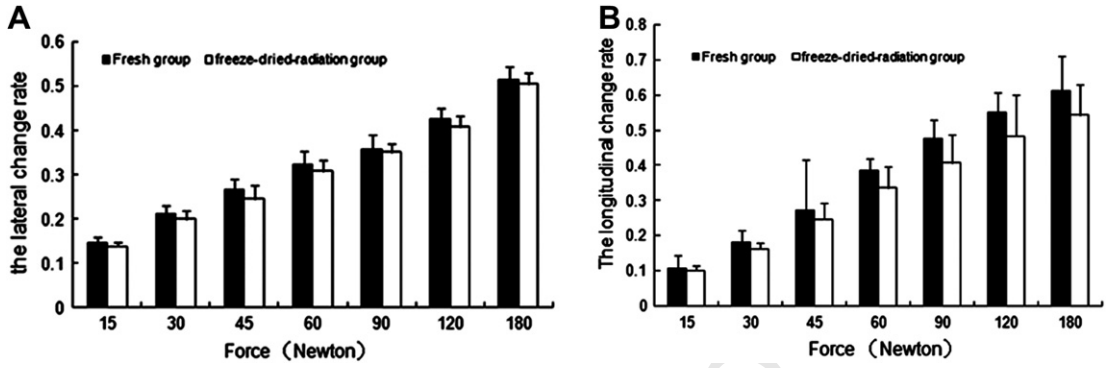

FIG. 3. Differences in physical properties between fresh vessels and freeze-dried radiation-treated vessels. (A) Lateral change rate; (B) longitudinal change rate). $P < 0.05$ *versus* the fresh group.

vessels digestion (Fig. 6A). ECs covered the vessels completely 2 h after implantation (Fig. 6B). After 9–12 d in culture, ECs covered the vascular inner surface and were tightly adhered (Fig. 6C). The number of implanted ECs was $3.62 \pm 1.25 \times 10^6$ in the freeze-dried radiation-treated allograft blood vessels, and the adherence rate was 90.98% ± 3.81%.

Observation of EC Inner Surface of Blood Vessels by Scanning Electron Micrographs

The animals used in this study were randomly selected to receive freeze-dried radiation-treated allograft blood vessels with or without an auto-EC lining. All incisions healed well and all animals gained weight during the postoperative period. Postoperative morphometric analysis of each allograft blood vessel revealed significant differences between the two groups. As assessed by scanning electron micrographs, few ECs were washed away to expose the smooth muscle or tissue under the intima during the early stage. Most ECs adhered tightly and the direction of cells dispose concomitantly changed in accordance with the force of blood stream. ECs gradually completely covered the intima following a prolonged period of time (Fig. 7).

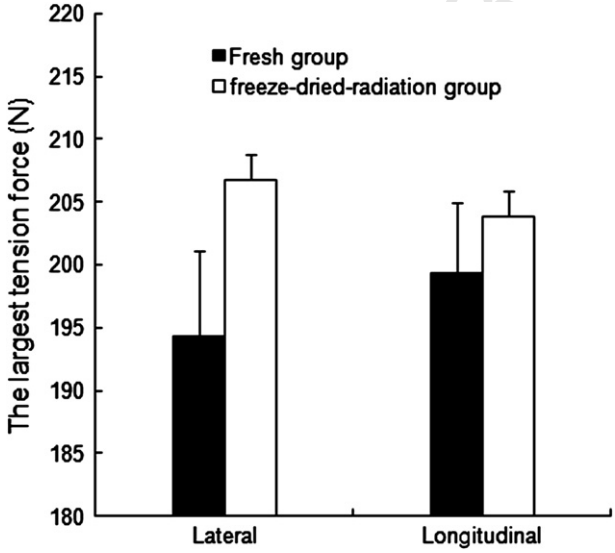

FIG. 4. The greatest tension force that the vessels could bear in the horizontal and longitudinal directions. $P = 0.001$ *versus* the fresh group in horizontal direction; $P = 0.045$ *versus* the fresh group in the longitudinal direction. The results indicate the freeze-dried radiation-treated vessels were stronger than the normal fresh vessels in response to tension.

Efficacy of EC Implants: Inhibition of Thrombosis and Intimal Proliferation

We determined *via* light microscopy that thrombi were observed at the sites of the vessels in the control group 1 wk after transplantation, and extensive neointimal proliferation and occlusive organized thrombi were observed in control animals after 1 wk. Particularly, the thrombi was significant organized in the 5 months and neovascular was observed in the organized thrombi. In contrast to the control animals, allograft blood vessels with implanted auto-ECs showed significant decreases in both restenosis and thrombosis. Neointimal hyperplasia was not obviously in EG and only slight neointimal hyperplasia was observed in 12 and 20 wk (Fig. 7). Angiography detected higher patency in the EG than CG (Fig. 8).

There were no differences in the amount of inflammatory cell infiltration earlier in 1 week, and more inflammatory cells were found around the suture. After 1 to 5 months, few inflammatory cells were detected or their numbers were obviously decreased in the graft vascular tissue with implanted auto-ECs, but in the control group there was no change in the numbers of inflammatory cells (Fig. 7).

DISCUSSION

The overall goal of these experiments was to determine the effects of re-endothelialization (by auto-EC

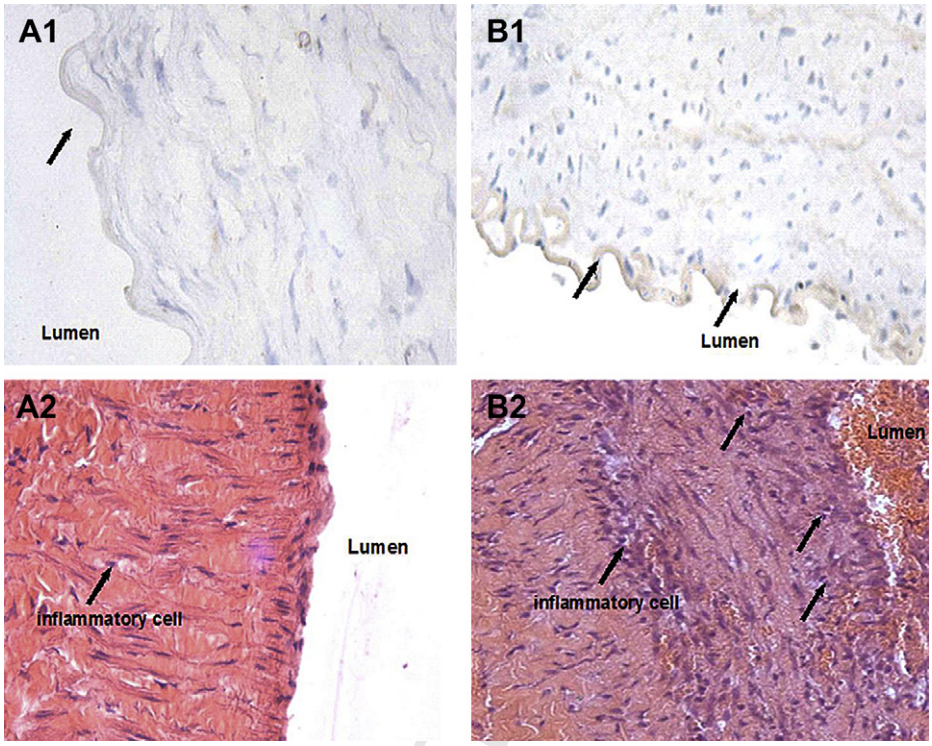

FIG. 5. Expression levels of MHC-II antigen and inflammatory reactions in freeze-dried radiation-treated vessels and normal fresh vessels. (A1) In freeze-dried radiation-treated vessels, yellow coloration was present in the elastic membrane and the expression level of MHC-II antigen was low (×400). (B1) In normal vessels, vascular ECs, the elastic membrane, and the whole texture had a brown coloration, and the expression level of MHC-II antigen was higher than that in the freeze-dried radiation-treated vessels (×400). (A2) ECs were present in the freeze-dried radiation-treated vessels that had been implanted with ECs, and there were fewer inflammatory cells (HE ×200). (B2) In the normal allogeneic vessels, the vascular internal surface was covered with red blood cells, and many inflammatory cells were present in the vessel wall (HE ×200).

seeding) on allogeneic vessels. The results demonstrated improved patency rates and reduced proliferative neointimal lesions in EC-seeded vessels. These observations provide further support for the notion that allogeneic vessels with implanted auto-ECs represent an important biological breakthrough in blood vessel transplantation.

The study of allogeneic vascular transplantation has revealed inevitable vascular graft rejection, especially for fresh allograft vessels. Much research has focused on methods to decrease the allogeneic antigenicity of vascular grafts, and many methods are available to reduce allogeneic antigens, such as cryogenic preservation, deep-freeze-drying and irradiation, glutaraldehyde treatment, multi-epoxide treatment, and glycerol treatment [8]. The antigenicity of aldo-vasculature is low, but its biological activity is also significantly reduced, such that it is not suitable for transplantation. It is unclear whether cryogenic preservation is useful in decreasing immunogenicity [9]. Freeze-drying and irradiation can significantly decrease the immunogenicity of vascular allografts, can prevent acute rejection [10], is inexpensive, easy to perform, and preserves the vessels' physiologic characteristics. In addition, Vesely

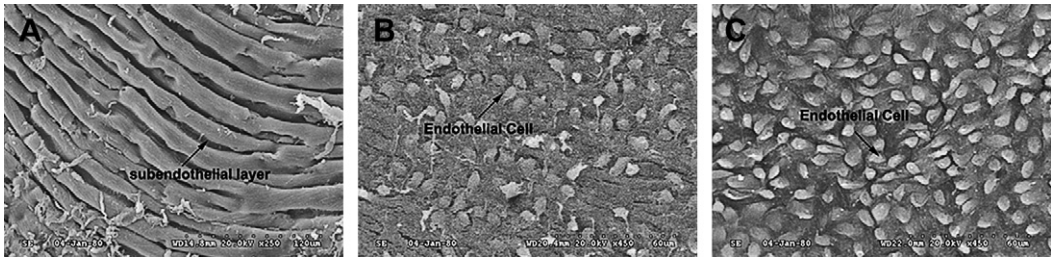

FIG. 6. Scanning electron micrographs of the vascular inner surface before and after EC implantation. (A) The smooth muscle was exposed in digested vascular inner membrane and no EC could be seen (SEM × 250). (B) Two hours after EC implantation, the cells covered the vessel completely (SEM × 450). (C) After 9–12 d in culture, the surface of the vessel was covered by ECs, and they were tightly adhered (SEM × 450).

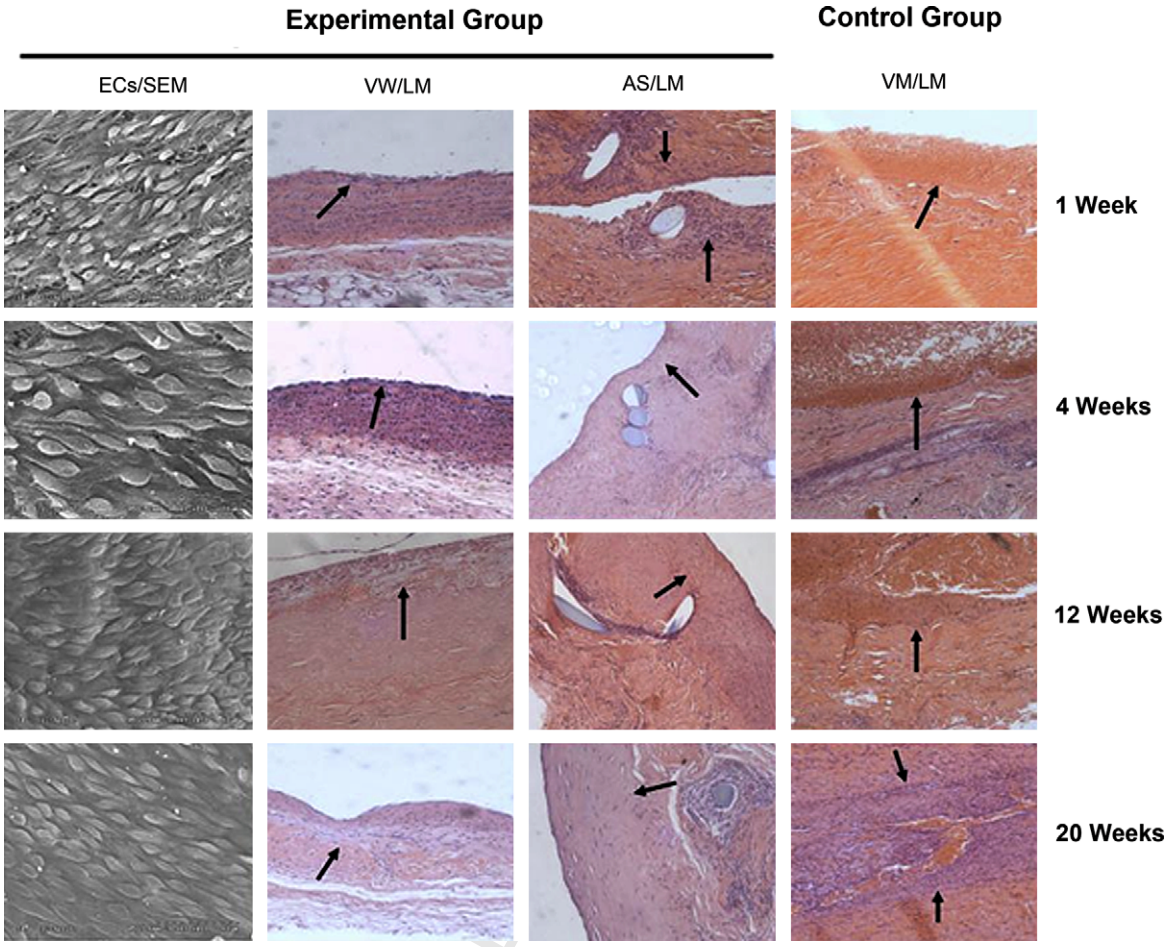

FIG. 7. Photomicrographs showing the coverage of ECs and the vein longitudinal sections about the effects of perivascular EC implants on allogeneic vascular grafts. Perivascular EC implants reduced intimal thickening (HE ×200). Arrows marking vascular inner membrane of the original vascular. VW: vessel wall; AS: anastomotic stoma; LM: light microscopy; SEM: scanning electron microscope.

found that irradiation can reduce antigenicity and preserve the physiologic characteristics of allografts [8]. In our study, we found that the compliance of the treated blood vessels was similar to the fresh blood vessels. But the greatest tension forces that the vessels could bear were different between the before and after treatments. We supposed that the treatment of freeze-dried radiation vessels may change the structure of collagen fiber but not elastic fibers. For this reason, the compliance of treatment vessels did not change and the tension maximum change obviously. Due to the low antigenicity [Q12] and excellent physiologic and hemodynamic characteristics of these grafts, lyophilized and irradiated allogeneic vessels can be regarded as the most suitable physiologic vascular material.

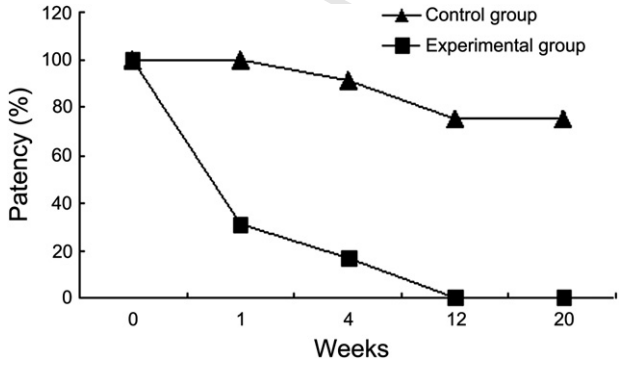

FIG. 8. Patency after transplantation detected by angiography. Significant differences were observed between the control and experimental groups.

The maintenance of the donor's endothelium is disadvantageous with regard to immunologic reaction in the recipient. ECs that present antigens are the first target for many immunologic reactions, and are subsequently destroyed. This can be further aggravated because allografts are not commonly transplanted in accordance with the ABO blood group system. Our study's purpose was to reduce the antigenicity of blood vessels and to reduce allograft rejection. Additionally, all of the treatments employed must maintain the vascular performance of the allografts. After comparing many approaches, the method of freeze-drying and irradiation was chosen because it greatly reduces rejection and maintains the allograft's physical characteristics.

We thus removed the ECs and used the method of freeze-drying and irradiation to reduce antigenicity.

The currently postulated mechanisms for thrombosis and intimal hyperplasia consist of three temporal phases that occur at different times after injury. An early phase, consisting of platelet activation and thrombus formation, occurs within minutes to hours, an intermediate phase of cellular recruitment occurs in hours to days, and a late proliferative phase occurs in days to months after injury. Inhibition of thrombosis does not eliminate smooth muscle cell proliferation or intimal hyperplasia, and the mechanism that controls the latter does not necessarily regulate the former. Only ECs can control all three stages. It has long been known that an intact endothelium provides control over a wide range of vascular biological activities, including thrombosis, vasomotor tone, smooth muscle cell proliferation and migration, lipid infiltration, and leukocyte adhesion, transmigration, and transformation [11–13]. ECs produce a myriad of factors that maintain vascular homeostasis [14]. Endothelial loss or damage disrupts vascular homeostasis and can result in proliferative neointimal lesions or changes in patency and occlusion rates.

Seeding allogeneic or xenogeneic ECs onto biological prostheses can regulate vascular repair, and has been described several times previously [5, 15, 16]. EC seeding has been extensively studied as a possible strategy for improving the long-term patency of small vascular grafts [17], and we also found a higher patency upgrade by ECs seeding. In studies on swine femoral arteries, Nabel *et al.* achieved 2% to 11% cell adherence after 30 min of reseeding, and in rabbits Thompson demonstrated 17% cell retention after 100 min [18]. The major challenges of EC seeding are the need to improve effective seeding and adhesion or to enable tight adherence in order to reduce cell displacement by the shear forces produced during blood flow. An increase in seeding density might be useful to increase its efficiency, but this requires large numbers of ECs. Omentally derived microvascular ECs can meet this requirement, but are also contaminated with non-ECs. Digestion of the vascular intima can provide pure ECs, but only in small quantities. In our preliminary experiment, we found that mechanical scraping and trypsin digestion could greatly injure the ECs. In this study, we followed the reported method to obtain ECs *via* collagenase digestion and reduced injury to ECs [19]. We cultured the ECs in a medium containing vascular endothelial growth factor. This treatment method accelerated cell growth and reduced the culture time. After implantation, we continued the culture for more than 1 wk to consolidate adhesion and enhance the ability of the cells to avoid becoming dislodged in response to blood flow. We assessed the levels of vWF in the medium to confirm that the normal functional characteristics of the ECs were maintained.

In summary, our results suggest that auto-ECs implanted onto allogeneic vascular grafts can resist blood flow and reduce thrombotic occlusion and intimal hyperplasia. ECs seeding resulted in an improved patency rate for small-diameter allogeneic vascular grafts in a mongrel vena jugularis model. Using manual methods for EC seeding, we obtained useful and reliable endothelialization of the small-diameter allogeneic vascular grafts.

Study Limitations

One limitation of this study is the long culture time to obtain enough auto-ECs, since many patients do not have such long waiting time. Further experiments are necessary to accelerate the growth rate or to obtain enough pure ECs. Another limitation of the study is that the function of transplanted ECs is unknown, and additional studies are needed to examine the function of these cells.

ACKNOWLEDGMENTS

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