Autologous Endothelialized Vein Allograft
A Solution in the Search for Small-Caliber Grafts in Coronary Artery Bypass Graft Operations

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Background—Early graft failure after the use of less satisfactory autologous grafts (30% of all vein grafts) is caused primarily by the following problems: (1) suitable autologous transplants are limited; (2) biotechnology has not yet been able to produce reliable graft substitutes that are legally and ethically approved; and (3) current prosthetic materials are prothrombotic. To overcome these problems, we developed an easily accessible, quality-controlled graft.

Methods and Results—Human autologous venous endothelial cells (HAVECs) were isolated from short segments of peripheral veins unsuitable for bypass grafting. After mechanical deendothelialization of cryopreserved allograft veins (CAVs) and precoating with recipient autologous serum, these homologous cells were seeded by use of a rotating device. Growth of a confluent HAVEC layer within 1 week in a special incubator was observed. After histological and mechanical tests, 12 patients received 15 grafts in total. Bypass operation was followed by clinical and angiographic follow-up. Production period was 22±8 days. HAVEC-coated CAVs showed normal connective tissue wall structure and a tight endothelial monolayer (burst pressure >2000 mm Hg). To date, 12 CABG patients lacking suitable autologous graft material have been treated. One patient died of a cause unrelated to the grafts, which were found morphologically normal and patent during autopsy. Of 15 grafts, 2 were occluded at the first angiographic follow-up. The oldest graft has now been in place for ≈3 years. Immune suppression was not administered.

Conclusions—At present, autologous endothelialized CAVs present good alternative small-caliber grafts for patients lacking suitable autologous vessels. (Circulation. 2001;104[suppl I]:I-108-I-114.)

Key Words: endothelium □ bypass □ coronary disease □ transplantation □ revascularization

The autologous saphenous veins and mammary arteries are at present the prime choice for use as grafts for aortocoronary revascularization. Limited availability of autologous bypass material and an increasing frequency of reoperations often necessitate the use of less satisfactory autologous graft material. In addition, it is a known fact that up to 30% of all saphenous veins used for grafting are of insufficient quality.1 In view of this, enormous efforts have been made over the last 30 years to produce alternative small-caliber grafts with acceptable patency rates.2 Synthetic materials such as polytetrafluoroethylene (PTFE) have been used successfully in peripheral vascular reconstruction but failed nonetheless when used for aortocoronary reconstruction.2 In the 1980s, attempts were made to establish cryopreserved allograft veins (CAVs) as bypass substitutes. Such allografts have in fact been used in patients without sufficient autologous graft material. Given the poor early and late patency rates, however, CAVs have not become widely accepted as bypass substitutes.2 Lately, several tissue-engineered grafts have been discussed1,3 and have yielded promising preliminary results although their clinical introduction is not likely to be implemented soon. Assuming that biotechnology would be able to produce a reliable graft substitute, it is improbable that it would not be confronted with massive legal and ethical objections.

In respect to its immense clinical importance, the search for an ideal small-caliber graft has been compared with the search for the holy grail.2 Forced by clinical necessities, we developed a new bypass graft using CAVs and recipient autologous endothelial cells. We used our experiences from endothelializing fibrin-coated ePTFE prostheses after the method of Zilla et al4,5 and modified this technique to suit the new task. The underlying principle for graft production lies in the autologous reendothelialization of a deendothelialized CAV with recipient cells (Figure 1). After establishing a simple, reliable, and reproducible production method of such grafts and the necessary quality assessment, we treated 12 otherwise inoperable patients with the new graft.
**Methods**

**Production and Validation of the New Graft**

The production procedure is shown in Figure 2.

**Preparation of CAVs**

Donor veins were harvested from organ donors according to German transplantation law and were cryopreserved according to protocol based on the guidelines of the American Association of Tissue Banks Standards for Tissue Banking. For graft preparation an, ABO blood group–compatible vein was thawed and mechanically stripped of its native endothelium by being pulled through a blown-up Fogarty catheter 3 times. Deendothelialization was verified by SEM (Leica S 420, Leica Microsystems AG) of inner graft surface samples.

**Isolation and Culture of Human Autologous Venous Endothelial Cells for Clinical Implantations**

Before the coating procedure, 300 to 500 mL blood was collected from the patient. The blood was stored at 4°C for 12 hours before the serum was obtained by centrifugation (10 minutes at 400g).

Human autologous venous endothelial cells (HAVECs) were isolated from a vein remnant of the leg (3 to 6 cm; caliber, 1 to 6 mm) of the patient according to the method of Jaffe et al. After the vessel was harvested, it was cannulated and rinsed with PBS. HAVECs were detached selectively from the vessel by a 30-minute incubation at 37°C in 0.1% collagenase D (Boehringer) in PBS supplemented with 1% human serum albumin and collected by flushing the vessel with a modified culture medium according to the method of Thornton et al (medium 199 substituted with 20% autologous serum, 5 to 10 ng/mL recombinant basic fibroblast growth factor, and 50 μg/mL heparin). The cells were washed twice by centrifugation (180g for 10 minutes), resuspended in culture medium, and then seeded in culture wells (20 000 cells/mL, 37°C, 5% CO2). When the cells became confluent, they were detached with trypsin/EDTA (0.05/0.02%) and passaged with a split ratio of 1:7. The medium was changed every third day.

**Preparation of the Grafts**

Once a sufficient number of HAVECs (~5×10^6 cells) was available, the deendothelialized CAV was then filled with the patient’s autologous serum and placed in an incubator at 37°C for ~12 hours. Subsequently, vein endothelialization was initiated by seeding 1.2×10^6 HAVEC/cm^2 inner graft surface in a rotating device (3 hours, 37°C, 9 rph) according to Zilla et al. Cell count was performed at the beginning of rotation and immediately afterward to quantify the adhesion rates of the seeded endothelial cells. After homogenous adhesion of the HAVECs to the inner graft surface had been achieved, the vein was placed in a special cultivation apparatus (Figure 3). The endothelialized vein was ready for implantation 6±1 days later.

**Verification of Homogenous Seeding of Endothelial Cells to the Inner Graft Surface of CAVs**

Six CAVs (measuring ~10 cm) were taken 6 days after reendothelialization and cut into samples measuring 1 cm each. The inner graft surface of the samples was evaluated by SEM.

**Biomechanical Stability**

**Burst Test**

Twenty native donor veins and 20 reendothelialized CAVs (free length, 5 cm; no branches) were burst by use of a high-pressure liquid pump and an electronic pressure sensor (Greisinger Meßtechnik). The Mann-Whitney test was used to analyze differences between groups. A value of P<0.05 was considered significant.

**Perfusion Test**

Ten reendothelialized CAVs were perfused at 37°C with medium 199 supplemented with 2% human serum albumin by a heart-lung machine (pressure, 80 to 100 mm Hg; flow, ~150 mL/min). Tissue samples were taken after 1, 3, 6, and 12 hours and evaluated by SEM.

**Analysis and Structural Comparison of the Extracellular Matrix of Donor Veins and Reendothelialized CAVs**

Specimens were fixed in 90% methanol. Cryosections (12 μm) of native veins (day 1) and reendothelialized CAVs were immunolabeled with monoclonal antibodies against glycosaminoglycan (kera-tan sulfate) and collagens (types I, III, IV, and V). The method for immunohistochemical labeling has been described previously in detail.

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**Image Descriptions**

**Figure 1.** Underlying principle. Cryopreserved vein allografts are mechanically deendothelialized and afterward reendothelialized with recipient autologous endothelial cells.

**Figure 2.** Flow chart of production process.

**Figure 3.** Cultivation device. Vein (1) in which autologous endothelial cells have already been seeded is maintained in medium-filled cultivation vessel (2). Ends of vein are attached to vessel outlets via adapters (3). Original distal vessel end is attached to medium-filled Boyle-Marriott bottle (4). Original proximal end leads to waste collection vessel (5). Pressure gradient, ΔP (6), which depends on the depth of cannula (7), is chosen to just prevent vein collapse. With aid of computer-controlled pump (8), medium in vein lumen is exchanged daily. Also shown are sterile filters (9) and access ports (10).
Quality Assessment of the New Graft

Criteria of Donor Veins

We used only donor veins measuring ≥10 cm that had no significant difference in caliber (diameter, 3 to 6 mm). For clinical implantations, ABO compatibility was an additional requirement. The processing, preservation, and storage of the donor veins were conducted according to American Association of Tissue Banks Standards for Tissue Banking.

HAVEC Culture

HAVECs were identified and characterized by phase contrast (cobblestone monolayer) and fluorescence microscopy (positive staining for factor VIII-Ag, CD 31, and uptake of acetylated LDL). For clinical purposes, HAVEC cultures were accepted only from the first or second cell passage and if the percentage of contaminating cells (e.g., fibroblasts, smooth muscle cells, pericyte-like cells) was <5%.

Endothelialization and Cultivation Procedure of the CAVs

An initial adhesion rate >90% was obligatory, as was daily cell count in the medium waste to exclude unexpected detachment of seeded HAVECs. Any kind of detachment was considered a contraindication for implantation. A tissue sample was taken for SEM 1 day before implantation for evaluation of inner graft surface. Microbiological tests performed several times throughout the whole procedure excluded bacterial and fungal contamination. The tests included tissue samples of the donor veins after initial harvesting and after thawing.

Clinic

All implantations were done with written informed consent of the patients and with approval of the ethics committee of the Ludwig-Maximilians University of Munich. We only included patients after saphenectomy or with severe varicosis diagnosed by phlebography. Patients who could be treated by use of a single radial artery were also excluded. Six patients needing hemodynamic monitoring were hospitalized throughout the production period, whereas the other patients were discharged 1 to 2 days after harvesting of the vein remnant. They were called in again when the respective grafts were ready. If an implantation was scheduled, the characterization of endothelial cells, an SEM of the inner graft surface, and various microbiological tests were considered obligatory. For the first 4 patients, the rejection of the patient by a cardiac surgical or a cardiological interventional unit was an additional requirement. Patient characterization is given in the Table.

Operation Technique

The harvest of a vein remnant was done 3 weeks before the CABG operation in patients under local anesthesia, and patients were sent to the regular ward immediately thereafter. The aorto coronary procedure was performed either on the beating heart (1 patient) or by use of the extracorporal circulation (11 patients) with standard surgical techniques. No special care was required when the vein graft was handled. Immunosuppressive agents were not administered.

Clinical Follow-Up

Clinical examinations were performed daily for the first 7 days by cardiac surgeons and afterward on routine visits by the respective cardiologists. To evaluate graft patency, the ethics committee allowed only 1 angiography in the first postoperative year if the patient was well. The time interval from operation to angiography was dependent on the comorbidity of the patients.

Results

Since 1997, we have produced a total of 98 reendothelialized CAVs according to the protocol. Fifteen grafts were used for clinical implantation; the other grafts were used for scientific purposes. The average production time of implanted grafts was 22±8 days.

HAVECs were characterized by a strictly single-layered cobblestone morphology and endothelial cell–specific staining for factor VIII-Ag, CD 31, and acetylated LDL uptake. Autologous serum was gained after incubation of the autologous blood samples for 12 hours at 4°C.

After the cryopreserved ABO-compatible allograft vein was thawed, the remaining native endothelium was totally removed in each case by pulling a Fogarty catheter through 3 times. Figure 4a shows an SEM of a deendothelialized CAV.

The initial adhesion rate (seeded cells minus nonadherent cells divided seeded cells) was 95±3%. During the cultivation procedure, we were in no case able to detect detached cells in the medium waste.

All reendothelialized veins showed an intact, vein-typical endothelium on the inner surface on SEM 6±1 days after
r endpointalization of the cryopreserved vein with recipient autologous endothelial cells in the cultivation apparatus (Figure 4b).

In all perfusion experiments, SEM showed only minimal differences in the endothelial cell coating of inner graft surface. Detachment of HAVECs was not visible. Figure 4c shows the inner graft surface of a reendothelialized CAV of a patient who died 48 hours after an CABG reoperation. The respective reendothelialized grafts were kept in the cultivation apparatus for 6 days before the operation. The SEMs demonstrate spindle-shaped alignment of the seeded endothelial cells in the direction of the blood stream.

Figure 5 shows the burst pressures of native donor veins and reendothelialized CAVs. There was no significant difference between native veins and reendothelialized CAVs. All veins were immunolabeled for collagen types I, III, IV, and V and for keratan sulfate at all stages of cryopreservation and 48 hours after implantation (Figure 6). Labeling for collagen type V was most prominent around the endothelial cells and was seen 48 hours after implantation. With increasing duration of cryopreservation and implantation, the initial homogeneity of the keratan sulfate labeling in the extracellular matrix decreased.

After 48 hours of implantation, the nuclei of seeded endothelial cells can be clearly detected on the inner surface of the extracellular matrix of the donor vein, which does not contain other nuclei in its deeper wall.

Clinical Results

Patient characteristics and clinical data are summarized in the Table. Twelve patients received the new bypass between January 1998 and November 2000. In all cases, it was possible to locate and harvest a vein remnant from the patients’ legs. The harvesting of remnants took between 20 and 80 minutes, depending on the quality. There were no postoperative complications (ie, wound healing disorders) caused by the vein harvest.

Eleven patients had already had ≥1 postoperative coronary angiogram; 1 patient, who is in good clinical condition, has so far refused an angiography. One patient died 48 hours after implantation, following an aortocoronary reoperation and suffering from postinfarction angina. This patient required the intraoperative insertion of the intra-aortic balloon pump and finally died of acute ischemia of the left leg. The prolonged procedure was complicated by the fact that both of his mammary arteries were too short for free transplantation and needed to be sewn together with pieces of the new graft. Autopsy revealed that the new grafts were patent and showed an intact endothelium.

In all other patients, 2 implanted grafts were found occluded 3 months after implantation, as were the right mammary artery grafts in the same patients. One right mammary artery was significantly stenosed after 13 months and needed to be dilated. All other grafts were open and nondilated after ≥3 months (Figure 7). All patients were in good health at their last follow-up.

Discussion

Cardiac surgery is one of the most cost-intensive disciplines of medicine and is more dependent on procedural perfection than many other operative disciplines. Although impressive progress has been achieved during the last 20 years in the perfection of the operative technique, there has not yet been a clinically relevant alternative bypass constructed for use in the aortocoronary position. The problem is even more dramatic when one considers that many patients who are lacking enough autologous bypass material are also suffering from additional conditions known to be graft failure–related (ie, reoperations, insulin-dependent diabetes mellitus, and obesity). Such patients are vitally dependent on the structural integrity of the implanted grafts. It is also known that saphenous veins taken during a reoperation have reduced patency rates compared with those taken during the first operation. Even when harvested during the first operation, the perioperative damage of the vein endothelium during harvest and intraoperative storage results in early occlusion rates of ≈15% within the first year after surgery. Because all attempts to use clinically implemented materials as alternative grafts in the aortocoronary position have failed, it is not surprising that tissue-engineered grafts have attracted the interest of many researchers. Early attempts to produce tissue-engineered blood vessels have been promising but are far from clinical introduction. We demonstrated the possibility of producing a clinically usable tissue-engineered graft by coating cryopreserved veins with patient autologous endothelial cells within ≈3 weeks. Moreover, the graft proved suitable for clinical implantation even in patients with high comorbidity.

To a large degree, cryopreservation and banking of human tissue and organs to conserve them for use at a later stage has become standard procedure. The use of cryopreserved venous allografts is established in bypass surgery and in patients who do not have sufficient or qualitatively suitable vessel material of their own. The use of mere CAVs shows poor longevity, perhaps because of immunologically conditioned degeneration of the graft. In addition, premature thrombotic occlusions are often observed. These 2 problems have been traced back to damage of the donor endothelium that took place during the cryopreservation process. This can
result in the total absence or limited functioning of the endothelium. As a result, cryopreservation techniques for allografts and xenografts have been published that aim to guarantee a high degree of preservation of the donor endothelium. The degree of preservation of the donor endothelium of cryopreserved tissue is given in the literature as 50% to 80%. However, a major role has recently been assigned to the endothelium as being the culprit for acute and chronic organ rejection. Endothelium-specific, non-HLA antigens that lead to the activation of CD4 T cells enables the donor endothelium to supply the recipient’s immune system with foreign antigens in conjunction with other accessory molecules. The release of non-HLA antigen by damaged endothelial cells leads to a chronic immune reaction and possibly to graft vasculopathy and chronic rejection. Paying heed to these considerations, we decided to totally remove the donor endothelium. Whereas vascular endothelium is characterized, for example, by numerous antiaggregatory, anticoagulatory, and profibrinolytic activities, the components of the deeper vessel wall (ie, deendothelialized cryopreserved vein allografts) are mainly characterized by the expression of tissue factor, which initiates an immediate coagulation reaction when coming in contact with plasma factors. Therefore, we decided to reendothelialize deendothelialized CAVs to improve the antithrombogenic features of those grafts.

Although commercially available adhesion molecules (ie, fibronectin with or without proteoglycans) were used in almost all published coating procedures, we decided against their usage. Apart from the fact that it is unknown what influence the high nonphysiological concentrations of these substances may have on the functional differentiation of tissue, their use is raising clinical and legal objections. In vitro studies that we carried out regarding the endothelialization of CAVs showed that a precoating of the veins with autologous serum presented an ideal matrix for the cell repopulation of inner surface of the veins. A precoating with the patient’s autologous serum in physiological concentrations promoted not only the adhesion but also the functional differentiation of the endothelial layer. Because the serum is totally autologous, there are no clinical or legal objections. To facilitate the reendothelialization of CAVs, we developed a 2-stage technique. The initial adhesion of the endothelial cells is performed with an already-known rotating device. Further cultivation is carried out by use of a special cultivation device characterized by its ability to create a constant pressure gradient between the lumen of the vessel and the outer area of the vessel. This prevents the walls of the vessels from collapsing. In addition, it specifically enables the nourishing of the seeded endothelial cells and the vein wall, as well as the outwash of unwanted metabolic products by transmural filtration. In contrast to other tissue-engineered grafts, the average production period of our graft is only 3 weeks. However, the mechanical stability is still comparable to that of native veins, which has been proved in burst pressure and perfusion tests. Immunohistochemical examinations have shown that the major components of the extracellular matrix responsible for its mechanical stability (ie, fibrillar collagen types I and III, keratan sulfate as a molecular part of various proteoglycans) are persisting through all stages of cryopreservation and tissue culture and even after implantation. The same result can be obtained for type IV collagen as a typical part

Figure 4. SEMs of inner graft surface. a, Vein after complete removal of native endothelium with Fogarty catheter (original magnification ×30). b, Same vessel after successful reendothelialization of entire inner surface with autologous saphenous vein endothelial cells. Note confluent but irregular endothelial cell lining (original magnification ×150). c, Autopsy sample. Vein explanted 48 hours after implantation. Note spindle-shaped alignment of confluent endothelial cell layer in direction of blood flow (original magnification ×75). Inset is at higher magnification (original magnification ×300).

Figure 5. Comparison of mechanical stability (burst pressure) of freshly harvested veins and reendothelialized CAVs.
of the basal lamina. The observation that seeded endothelial cells produce type V collagen, which is regarded as an important regulating factor for cell adhesion in wound healing,\(^{25}\) may be indicative of the necessity for a specific composition of the underlying extracellular matrix to assist endothelial growth. This essential environment for endothelial growth is best met with the use of CAVs, because this material maintains much of its unique extracellular matrix composition.

Figure 6. Immunohistochemical labeling for collagens (a through h) and keratan sulfate (i and j) in vessel wall. Left row shows appearance of native vein at day 1 after harvesting; right side shows cryopreserved allograft vein after 8 days of reendothelialization (and after implantation into patient for 48 hours). All scale bars, 20 \(\mu\)m. a through d, Widespread, diffuse labeling for type I and III collagen in vessel wall does not show significant differences between 2 groups. a, Labeling for type I collagen 1 day after harvesting; b, labeling for type I collagen 2 days after implantation; c, labeling for type III collagen 1 day after harvesting; d, labeling for type III collagen 2 days after implantation. e and f, No difference in labeling for type IV collagen can be observed 2 days after implantation. e, Collagen type IV 1 day after harvesting; f, collagen type IV 2 days after implantation. g and h, Labeling for type V collagen is detected around endothelial cells (arrows). Remember that cells on right side (h) are not donor but host cells that have been transplanted for 2 days; left side shows donor cells at day 1 after harvesting (g). i and j, Widespread and diffuse labeling for keratan sulfate in vessel wall. At day 2 after implantation (j), homogeneity of keratan sulfate labeling decreases compared with day 1 after harvesting (i).

Figure 7. Representative coronary arteriograms of autologously endothelialized venous coronary artery bypass grafts 6 month after operation. Arteriograms show patent, nondilated allografts. a, Patient 4; b, patient 6.
When ready for implantation, the suturability of the new graft is in no way different from that of native veins. No additional considerations are necessary. Because only organic tissue is involved, a higher resistance to infection compared with synthetic grafts can be postulated. This view is strengthened by experiences gained from allografts implanted in the aortic valve position. Our first clinical results are very promising and demonstrated good patency rates, especially when one considers the comorbidity of our patients. Notably, there has been a combined occlusion of the new graft and the right mammary arteries in the same 2 patients, possibly caused by non-graft-related conditions. On the other hand, insulin-dependent diabetes mellitus and chronic hemodialysis do not seem to be contraindications for the new graft. We believe that there is an important immediate need for an alternative graft in the aortocoronary position. The first patient we treated was considered to be inoperable because of a lack of graft material by 2 cardiac surgical units although she was suffering from significant left main artery stenosis. She was in our cardiological intensive care unit and had been reanimated repeatedly but subsequently refused any further CPR. As a last resort, we offered the implantation of the new graft for which we did not see an ethical objection. The worst-case scenario was the accidental detachment of the seeded endothelial cells, thus leaving a deendothelialized allograft vein in place. In addition to the above considerations,20 –22 animal experiments maintain that deendothelialized allograft vein in place. In addition to the above considerations,20 –22 animal experiments maintain that deendothelialized allografts produce better patency rates than intact allografts.23 On the basis of these considerations, the ethics committee of our university did not see the necessity to indulge in animal implantations and subsequently permitted us to use this new graft on our patients (4 of whom were considered untreatable by cardiac surgeons and cardiologists). We conclude therefore that the cryopreserved and autologous endothelialized allograft vein is a new tissue-engineered alternative graft that offers the necessary clinical safety for those patients who lack autologous material. The short production period should help to facilitate broader clinical use. The graft should also prove useful in peripheral reconstructions. Thus, this graft may indeed be an answer to the search for a smaller-caliber graft in CAGB operations and thus may allow the treatment of many patients who otherwise would be deemed inoperable.

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