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ENDOTHELIAL CELL SEEDING: REVISITING THE ISSUES

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In the infrainguinal position, the primary patency of synthetic vascular grafts lies between 30% and 55%.[1-3] This disappointing outcome is opposed by a 5-year primary patency of approximately 70% for saphenous vein grafts in a comparable patient group.[1] As a consequence, endothelial seeding has been introduced with the goal to reduce the surface thrombogenicity of the prosthetic material through the creation of an autologous endothelial coverage. First attempts were based on a single-staged technique, where freshly harvested venous endothelial cells were seeded. In clinical trials, however, single-staged seeding failed, most likely because of the low number of available endothelial cells and the resulting insufficient endothelial cell inoculum.[4]

In the 1980s two alternative techniques were developed that addressed this shortcoming: endothelial cell sodding[5] and in vitro endothelialization.[6] While sodding exploits the abundance of microvascular endothelial cells in adipose fat tissue, in vitro lining achieves a similar goal through mass culture of venous endothelial cells. Our group has been involved with in vitro lining over the past 15 years.[6-11] The present report summarizes almost 11 years of clinical experience with close to 140 patients and describes our efforts to enhance graft performance in current clinical studies.

Patients and methods

With the clinical introduction of a new and untested surgical treatment for peripheral artery disease, our human trials had to be guided by three principle considerations: First, we had to initially assume that vein grafts are superior to endothelialized ePTFE grafts before proved otherwise. Therefore, the study was confined to patients who had no venous conduit available. Second, the standards established by Rutherford for studies involving new methods in vascular surgery required that only a limited number of patients be initially randomized. We restricted the randomization of the first phase of our study to less than 50 patients. The third determinant for the design of the study was the fact that autologous endothelial lining involves a time-dependent in vitro procedure. Thus, our study was naturally restricted to patients with subacute or chronic peripheral occlusive disease.

When the 3-year follow-up of the randomized phase 1 of our trial, as well as a similar study from another center, showed significantly better results in the endothelialized group than in the control group, ethical considerations determined phase 2. Since the withholding of the treatment from patients with no saphenous vein available would not have been justified against the background of the phase 1 results, we decided to offer the treatment as a routine procedure to all nonacute patients who relied on ePTFE prostheses. Eventually, after 10 years of clinical phase 1 and 2 trials including 152 in vitro endothelialized ePTFE grafts, it became safe to conclude that in vitro endothelialized ePTFE grafts perform similarly to autologous vein grafts. As a result, phase 3 of the study was initiated as a European multicenter study in which endothelialized ePTFE grafts are being compared with saphenous vein grafts in the challenging below-knee position. Prior to the commencement of this randomized multicenter study, we aimed at the further improvement of the shear stress resistance of the lined endothelium through the use of an RGD-enriched, engineered fibrin matrix as a precoating substrate.

Phase 1

From mid 1989 and onward, 49 patients were randomized. Details of this study were previously reported.[9] Indications for surgery were disabling claudication in 37 patients (grade I chronic limb ischemia) and chronic limb ischemia in 12 patients (grade II in six patients and grade III in six patients). For endothelial cell harvest, the right external jugular vein was taken under local anesthesia. Mass culture of first-passage endothelial cells was performed with first-passage endothelial cells until the required

cell number of 16 × 106 endothelial cells was confirmed by in situ counting. Reinforced 6-mm ePTFE grafts (W.L. Gore, Flagstaff, Ariz) were precoated with clinically approved, fibrinolytically inhibited fibrin glue (Immuno, Vienna, Austria) and were further coated with human fibronectin (28 μg/mL). Seeding of endothelial cells onto the graft surface (8.1 + 4.2 × 105 EC/mL cell suspension) was done in an automated, temperature- and pH-controlled rotation device (Microplan, Vienna, Austria).[6,11] Subsequently, grafts were incubated for another 11.6 + 3.0 days to allow the maturation of the cytoskeleton. Follow-up studies were performed after 9 days, 3 months, 6 months, and 1 year; they were performed on an annual basis thereafter. Patency was determined by the ankle/brachial index (ABI) and duplex sonography at each time interval. Angiography was performed annually and in patients with suspected graft occlusion. Graft occlusion was suspected if a deterioration of the clinical status of the limb was confirmed by a significant drop in the ABI or a lack of flow signal on duplex sonography. Only primary graft patency was considered. During the entire observation period, none of the patients were lost to follow-up. Eight patients in the control group and 10 patients in the endothelialized group died during follow-up for reasons other than peripheral vascular problems.

Phase 2

After June 1993, in vitro endothelialization was offered to all patients undergoing bypass operations for peripheral arterial occlusion who were lacking a suitable saphenous vein. One hundred twelve patients received 125 successfully endothelialized ePTFE grafts; in 13 of these patients an endothelialized prosthesis was bilaterally implanted. One hundred two graft implantations were done for clinical grade I ischemia (76 above knee, 26 below knee), 13 for grade II (9 above knee, 4 below knee), and 10 for grade III (6 above knee, 4 below knee). Because of the unavailability of human fibronectin for clinical use during phase 2, neither culture vessels nor grafts could be preincubated with it. ABIs and duplex sonography were performed at 1 month, 3 months, 6 months, and 1 year; from then onward, they were performed on an annual basis or at any deterioration of the clinical status. Angiography was only done after 1 year and on suspicion of graft occlusion. No patient was lost to follow-up, and 11 patients died of unrelated causes.

Statistical analysis of the 78-month follow-up of our randomized trial (mean observation period 60 months) and the 72-month follow-up of the subsequent routine clinical implantation of endothelialized grafts (mean observation period 20 months) was performed with STATISTICA (StatSoft, USA) software using the Kaplan-Meier survivorship function for survival analysis and the log-rank test and Gehan's Wilcoxon group comparison.

Phase 3

1. Improvement of precoating substrate: First- or second-passage adult venous endothelial cells were confluently seeded onto 15-cm long precoated ePTFE vascular grafts as described elsewhere. Precoating was done either with the standard conventional fibrin matrix also used in phase 1 and 2 of the clinical study or with an engineered fibrin that used the factor XIII cross-linking process for the inclusion of RGD peptide into the matrix (3.5 mg/mL fibrinogen stock solution; Tisseal; Baxter/Immuno, Austria).[12] After seeding, grafts were postcultivated for another 9 days to allow the maturation of the cytoskeleton. Subsequently, endothelialized graft segments were placed into a perfusion loop that emulates the pulsatile flow patterns and shear forces of femoral arteries while sustaining physiologic metabolic conditions for the cells.[13] Four endothelialized grafts in each group were exposed to such shear stress conditions for 24 hours. Samples were analyzed by electron microscopy, histology, and vital epifluorescence microscopy before and after shear stress exposure. Epifluorescence images were digitized with a Coolpix 950 digital camera (Nikon, Japan) attached to the microscope. Digital micrographs were then analyzed with Photoshop (Adobe, USA) software, allowing the computation of the percentage of cell loss. Thrombogenicity studies and a preclinical confirmation of the in vitro experiments are currently under way in the nonhuman primate model.
2. European Multicenter Study. Applications to the institutional ethics committees either have been approved or are currently under review. Each center has a randomization protocol for initially 100 patients, comparing endothelial-lined ePTFE grafts with saphenous vein grafts in the below-knee position.

Results and discussion

The 152 grafts reported in both phase 1 and 2 of our clinical study represent the same ratio of above- versus below-knee grafts as the vast majority of studies reported in the literature. As far as clinical stag-

ing is concerned, the literature is inconclusive. Although often implied that the prognosis of stage III patients is worse than of stage I patients, practically all large multicenter studies did not distinguish between clinical stages.[11] However, if one compares ePTFE studies, in which most patients were in stage III with others that only investigated stage I patients, it seems as if the average 5-year patency of ePTFE prostheses lies in the mid-50% range for stage I patients and in the upper 40% range for stage III patients.[11] Moreover, most of these studies were done in patients in which a vein graft was theoretically obtainable. In a group that—similar to ours—had no vein graft available, Veith et al[1] reported a 5-year patency of 29% for ePTFE prostheses

In our phase 1 randomized trial, the Kaplan-Meier survivorship analysis showed a primary 3-year patency rate of 84.7% for endothelialized grafts and 55.4% for control grafts. After 5 years it was 73.8% for the endothelialized group and 20.8% for the controls. After 9 years, the primary patency rate for endothelialized grafts remained high at 65%. Performance differences between the two groups were statistically significant (log-rank test; P = .00098 and Wilcoxon test; P = .0025).

The subsequent phase 2 routine clinical implantation of endothelialized ePTFE grafts showed a 6-year primary patency rate of 64.4% for all femoropopliteal reconstructions. The 90 grafts implanted in above-the-knee position during phase 2 had a 5-year patency of 62.0%, which compares well with Veith's 70% for above-the-knee saphenous vein grafts. The 3-year primary patency rate for our 35 endothelialized below-the-knee grafts was 78% comparable with the 80% that Veith et al reported for vein grafts in this position. The reversal of the normal patency pattern of ePTFE grafts with better results in the above-knee group is hypothesized to be due to a higher cell loss in this higher runoff and thus higher shear force group.

As a solution, an RGD-enriched fibrin matrix was tested at the onset of phase 3. In grafts precoated with the conventional fibrin formulation, cell loss in vitro was 37.7% ± 10.9%, corresponding with previous results. In contrast, cell loss was only 12.7% ± 12.0% in grafts precoated with the engineered, RGD-enriched fibrin matrix (P < .0001). Moreover, grafts precoated with the conventional fibrin matrix were also less confluently endothelialized (93.2% ± 5.2%) after 9 days of incubation than grafts coated with the fibrin containing additional RGD peptides (97.2% ± 2.8%; P = .025).

In summary, our patency results for ePTFE grafts that were lined in vitro with autologous endothelial cells were distinctly better than both our control grafts and the patencies reported for untreated ePTFE grafts in the literature. Furthermore, it appears as if in vitro endothelialization achieves an approximation of the patency of ePTFE grafts to that of vein grafts. In this regard our patencies of 85%, 74%, and 66% after 3, 5, and 9 years in phase 1 and 73% and 68% after 3 and 5 years in phase 2 compare well with the 3- and 5-year patencies of 75% and 68% for vein grafts in Veith's multicenter study.[1] The overall slightly higher patency rate for our in vitro lined grafts may be explained with the higher proportion of stage II and III patients in Veith's study. These results, together with an improved immediate cell retention on the engineered fibrin matrix, make it highly likely that the recently initiated multicenter study will eventually show a higher patency rate for the tissue-engineered ePTFE grafts than for vein grafts, even in the challenging below-knee position.

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VASCULAR TISSUE ENGINEERING: STRUCTURE VERSUS FUNCTION

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The blood vessel is a structure of extraordinary sophistication. The physical continuity of vascular cells and tissues that make up the blood vessel wall contribute to its structural integrity while also maintaining homeostasis through biochemical regulation. The endothelial monolayer that lines the normal blood vessel serves as a twofold regulator of vascular physiology. The endothelium provides structural integrity to the blood vessel by forming a continuous, selectively permeable, thromboresistant barrier between circulating blood and the arterial wall. At the same time, it also produces and supplies products that control blood flow, vessel tone, thrombosis, platelet activation, adhesion, aggregation, leukocyte adhesion, monocyte infiltration, and smooth muscle cell migration and proliferation. Endothelial injury, such as occurs after angioplasty, not only removes the physical barrier but also interferes with the biochemical regulatory potential of the blood vessel and sets into motion a sequence of events that leads to the proliferation and migration of smooth muscle cells resulting in obstructive arterial lesions. This process of restenosis leads to critical narrowings in significant numbers of patients after angioplasty, bypass grafting, and organ transplantation.

Tissue engineering enables the development of biological substitutes that restore, maintain, or improve tissue function[1] while also providing substrates by which to examine structure-function relationships for specific tissues or organs. Cells may be implanted at sites distant or in different configurations from their original state, enabling examination of any added potential benefit of cell secretory function to regulation of tissue biology above that imposed by preservation of tissue architecture alone. This might be especially important in vascular biology where the autocrine, paracrine, and endocrine function of the endothelium is rapidly emerging. Innovative studies have attempted to recreate the structure of the blood vessel by autologous endothelial cell transplantation, implantation of endothelial cell-seeded interposition grafts, or endovascular stents.[2-6] Yet, the question remains as to whether reestablishing biochemical control of vascular homeostasis also requires reestablishing the ordered architecture of the blood vessel. We have demonstrated, through the use of tissue-engineered endothelial cells, that the biologic effect of these cells on blood vessel regulation is maintained even when they are implanted at a distance from the lumen. Engrafted endothelial cells on three-dimensional polymer matrices, implanted in the perivascular space of injured rat carotid arteries, reduced intimal thickening by 88%.[7] In contrast, the isolated infusion of heparin, an endothelial cell product analog and potent smooth muscle cell inhibitor, only reduced intimal hyperplasia by 30%. These experiments supported the hypotheses that endothelial control over vascular repair is derived from the sum of a number of secreted endothelial cell–based products and need not emanate from the luminal surface.[8]

We recently addressed two important questions relating to the biologic effects of perivascular endothelial cell implantation. First, we examined whether allotransplantation of endothelial cell grafts was effective in controlling vascular repair in a porcine carotid artery model of vascular injury, thought to be less responsive to growth-regulatory agents than simpler animal models. Second, we explored whether xenotransplantation, a central issue in developing safe and practical clinical strategies, was more or less effective than allotransplantation. We now report that perivascular tissue-engineered endothelial cell implants exert profound control over intimal growth after arterial injury in pigs. Furthermore, despite an increased immune response to cross-species transplantation, beneficial control of vascular repair was maintained. These results provide insight into how the endothelium controls vascular homeostasis and are a further step toward the development of clinically viable strategies for modulating vascular repair after injury. Further exploration of how tissue-engineered endothelial cell grafts control vascular repair, particularly in settings of more chronic vascular injury, will afford insight into the structure and function of the blood vessel wall and into how experimentally effective techniques may be brought to clinical fruition.

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