From the Society for Vascular Surgery

Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts

Manfred Deutsch, MD, a Johann Meinhart, PhD, a Peter Zilla, MD, PhD, a Norbert Howanietz, MD, a Michael Gorlitzer, MD, a Alexander Froeschl, MD, a Andreas Stuempflen, MD, a Deon Bezuidenhout, PhD, a and Martin Grabenwoeger, MD, a Vienna, Austria; and Cape Town, South Africa

Objective: Based on a previous randomized study showing significantly superior patency rates for in vitro endothelialized expanded polytetrafluoroethylene (ePTFE) grafts we investigated whether it was feasible for a nontertiary institution to offer autologous in vitro endothelialization to all elective infrainguinal bypass patients who had no suitable saphenous vein available.

Methods: Over a period of 15 years, 310 out of 318 consecutive nonacute patients (age 64.7 ± 8.6) received 341 endothelialized ePTFE grafts (308 femoropopliteal: 153 above knee [AK] and 155 below knee [BK] and 33 femorodistal). Autologous endothelial cells were harvested from short segments (3.9 ± 1.1 cm) of subcutaneous veins (80% cephalic, 11% basilic, 2% external jugular, and 7% saphenous) and grown to mass cultures within 18.9 ± 4.5 days before being confluently lined onto fibrin glue-coated ePTFE grafts. The graft diameter was 6 mm (64%) or 7 mm (36%). The overall procedure-related delay for graft implantation was 27.6 ± 7.8 days. Growth failure prevented 2.5% of patients from receiving an endothelialized graft. The mean observation period was 9.6 years. Primary patencies were obtained from Kaplan-Meier survivorship functions. Explants for morphological analysis were obtained from eight patients.

Results: The overall primary patency rate of femoropopliteal grafts was 69% at 5 years (68% [AK] vs 71% [BK]) and 61% at 10 years (59% [AK] vs 64% [BK]). Primary patency of 7 mm vs 6 mm grafts was 78%/62% at 5 years and 71%/55% at 10 years. The difference between the two groups was statistically significant (log rank test \( P = .023 \); Breslow test \( P = .017 \)). Stage I vs II/III patients showed 5-year patencies of 67% vs 73% (N.S.) and 10-year patencies of 61% vs 53% (N.S.). The primary patency of femorodistal grafts was 52% at 5 years and 36% at 10 years. The limb salvage rate was 94% (fempop) vs 86% (femdistal) at 5 years and 89% vs 71% at 10 years. All retrieved samples showed the presence of an endothelium after 38.9 ± 17.8 months.

Conclusion: Autologous in vitro endothelialization was shown to be a feasible routine procedure at a nontertiary hospital. Explants confirmed the presence of an endothelium years after implantation while the primary patency in the particularly challenging subgroup of patients without a suitable saphenous vein resembles that of vein grafts. (J Vasc Surg 2009;49:352-62.)

Thirty years ago vascular surgeons pioneered the field of tissue engineering by attempting to create endothelialized synthetic bypass grafts. The underlying motivation for these efforts was the rather disappointing clinical performance of small- to medium-sized vascular prostheses. With the concept of “seeding” autologous endothelial cells into the meshwork of synthetic grafts vascular surgeons such as Malcolm Herring1 and Linda Graham2 laid the foundation for the broad array of today’s tissue engineering efforts across the disciplines. Initial enthusiasm was great, and many other groups successfully confirmed the concept under experimental conditions. Premature clinical studies, however, disappointed3,4 and eventually contributed to the termination of most of the programs in the 1980s. In search for an explanation for the discrepancy between animal studies and clinical trials, insufficient seeding densities on the many times longer clinical grafts seemed plausible. With mass-harvests of endothelial cells from fat tissue in its early stages5 mass-culture offered an alternative to the minuscule primary harvest from subcutaneous vein segments.6-8 Yet, in the absence of precedents, regulatory concerns prevailed and most of the pioneers of the first hour shied away from applying tissue culture techniques. Typically, those few institutions that proceeded with a cell culture-based approach were European,9,10 partly due to a less stringent regulatory environment, and partly due to health institutions that were more likely to accept the additional costs associated with “in vitro” endothelialization than their American counterparts. In the second half of the 1980s, sufficient experimental data had been accumulated by a few groups to justify the commencement of three independent clinical pilot studies.9,11 In contrast to the “single staged” seeding trials of previous years, in vitro endothelialization significantly improved clinical patency rates.9,11-13 Adding weight to these rather small clinical studies was the fact

352
that retrieved samples demonstrated the presence of a confluent endothelium in the midgraft region weeks to years after implantation.

As the group representing the main proponents of the treatment, we continually pointed out that it was rather the principle than the specific approach that we sought to prove. By choosing the particularly challenging group of patients who had no saphenous vein available, a long-term cohort was accumulated and followed up that had no autologous alternative. By offering the procedure to all qualifying patients at a nontertiary institution, we intended to demonstrate that cell culture-based treatments are feasible at the level of a community hospital.

PATIENTS AND METHODS

Following a controlled, randomized IRB approved trial between 1989 and 1991, the present cohort of patients was treated on the basis of informed consent for the “Medical Prescription of Autologous Cells” as stipulated under Austrian and European Union regulations.

Between June 1993 and December 2007, 570 consecutive patients required an infrainguinal bypass graft without having a suitable saphenous vein available. The lack of a suitable saphenous vein was a prerequisite for being considered for in vitro endothelialization. Of these patients, 190 (33%) had an acute indication for surgery and would therefore not have tolerated the delay associated with in vitro endothelialization. Another 69 patients (12%) were either operated by surgeons who chose untreated over endothelialized expanded polytetrafluoroethylene (ePTFE) grafts (2/3 surgeons in 1993 gradually decreasing to 0/5 surgeons) or defaulted into this group due to growth failure. One patient refused consent. Of the remaining patients 310 out of 318 assigned patients (55%) (203 male and 107 female; mean age 64.7 ± 8.6 years) received in vitro endothelialized ePTFE grafts. Since 33 patients received endothelialized prostheses bilaterally the overall number of in vitro endothelialized ePTFE grafts implanted in this second enrolment since 1989 was 341. The reason for a lack of suitable saphenous vein was varicosity in 32%, insufficient diameter of <3 mm at the preoperative ultrasound assessment in 42% and the previous harvest of both saphenous veins in 26%. Of the 341 bypass grafts, 308 were femoropopliteal (153 above knee and 155 below knee) and 33 were distal reconstructions of which seven received an additional vein-bridge graft using saphenous vein segments (as the overall length of the saphenous vein would have been too short for using it as the main graft). Grading of ischemia was done in accordance with the Rutherford staging system.15,16 In 224 cases, graft implantation was done for grade I ischemia (114 above knee, 101 below knee, 9 distal reconstructions), 39 for grade II (14 above knee, 16 below knee, 9 distal reconstructions), and 68 for grade III (25 above knee, 38 below knee, 15 distal reconstructions).

Procedural development. Like other tissue engineering approaches for clinical applications our in vitro endothelialization techniques experienced a gradual improvement over the past 20 years. Especially, the construction of an easy-to-use and safe rotation device led to an improved and reliable seeding quality. Similarly, the optimization of cell culture reagents and culture conditions substantially shortened the time it took to produce a confluent endothelialized graft. Particularly, the tight monitoring for risk factors, especially lipids, prior to culturing of endothelial cells led to a significant reduction of growth failures.17

Fig 1. Immune-fluorescent live-dead stains of control samples taken immediately after seeding. Overall, 78% of the grafts showed complete confluence (a), 16% areas of preconfluence (b), and only 6% had minor endothelial-free areas (c).
Other methods like fibronectin precoating and the rescue of growth impaired cultures through exchange of the serum supplement of the culture medium had to be abandoned in 1994 because of the unavailability of GMP produced reagents. Since many patients were already referred on statins, risk-factor profiles for lipids were downward biased during the second half of the program. With the adoption of 7 mm prostheses in 1997, a further variable was introduced.

**Endothelial cell harvest.** The cell culture laboratory and the operating room were in close proximity. After sterile draping of the operating field, short segments of vein (3.9 ± 1.1 cm) were harvested under local anesthesia (Cephalic vein [80%]; basilic vein [11%] and external jugular vein [2%]). In 7% of the cases, a segment of the great saphenous vein was used when the vein was not sufficient for autografting and/or other veins were not suitable. Starch-free gloves were principally used to avoid the cytotoxic effect of glove powder. After no-touch dissection of the vein segments and in situ cannulation, the veins were flushed with medium 199 (Gibco, Paisley, UK; 10 ml, 37°C), filled with 0.1% collagenase solution (Worthington Biochemical Corporation, Freehold, NJ) (37°C) and kept distended between stop-cocks. The collagenase-filled vein segments were then transferred to the laboratory in phosphate-buffered saline solution (PBS) (37°C) in a sterile thermo-container. After 15 minutes of collagenase exposure cells were harvested by flushing the vein segment with M 199 (containing 20% of autologous serum) and collecting the cell-containing suspension in a sterile tube.

**Endothelial cell cultures and graft lining.** Primary cultures were grown in T 12 filter-protected culture flasks (Falcon, Franklin Lakes, NJ). Successfully grown primary cultures were passaged at pre-confluence into two 162 cm² culture flasks (Costar, Cambridge, Mass). A microgrid technique enabled the daily in situ quantification of available first-passage endothelial cells. Mass cultures were continued until the required cell number of approximately 18 × 10⁶ endothelial cells per graft was reached. The culture medium consisted of medium 199 supplemented with 10 ng/mL recombinant bFGF (Boehringer, Ingelheim, Germany; in phase 2), 50 mg/mL gentamycin (Gibco, Paisley, UK), 5 mL Fungizone (Sigma) and 20% of autologous serum. Prior to endothelial cell lining, expanded PTFE grafts of 70 cm length were precoated with fibrinolytically inhibited fibrin glue (Baxter, Vienna, Austria) and filled with the endothelial cell suspension (9.8 ± 3.4 × 10⁵ EC/mL culture medium). All ePTFE grafts were ring-reinforced. An even surface distribution of endothelial cells was achieved through a microprocessor controlled seeding device (Biegler Electronics, Vienna, Austria) rotating at 6 rph at 37° for 5 hours, providing a 5% CO₂ atmosphere. After seeding, grafts were left in culture medium for approximately 9 days to allow the maturation of the cytoskeleton of endothelial cells and thus increase their shear stress resistance.

**Graft implantation.** While the grafts were anastomosed to the arteries, the culture medium was kept-inside the prostheses by tilting the operating table without ever clamping the endothelialized graft the excess length was later cut-off. Prior to completion of the distal anastomosis, the culture medium was flushed out of the graft. Thin-walled ePTFE (W.L. Gore & Associates, Inc, Flagstaff, Ariz) was used as graft material. The initial 70 patients received a graft with 6 mm inner diameter. From then onwards, the graft diameter was chosen to be either 6 mm or 7 mm depending on target-vessel dimensions and run-off. All patients received anti-aggregatory treatment (oral dipyridamole, 75 mg/d and oral acetyl-salicylic-acid 330 mg/d given as combination drug

<table>
<thead>
<tr>
<th>AK</th>
<th>Mean/total</th>
<th>Age</th>
<th>Male</th>
<th>Female</th>
<th>Smoker</th>
<th>Nonsmoker</th>
<th>Ex-smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.8</td>
<td>108</td>
<td>45</td>
<td>88</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>±9.1</td>
<td>70.6</td>
<td>29.4</td>
<td>57.5</td>
<td>7.8</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td>BK</td>
<td>Mean/total</td>
<td>69.1</td>
<td>96</td>
<td>59</td>
<td>86</td>
<td>9</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>±8.3</td>
<td>61.9</td>
<td>38.1</td>
<td>55.5</td>
<td>5.89</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>Mean/total</td>
<td>68.7</td>
<td>22</td>
<td>11</td>
<td>13</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>±8.8</td>
<td>66.7</td>
<td>33.1</td>
<td>39.4</td>
<td>6.1</td>
<td>54.5</td>
<td></td>
</tr>
<tr>
<td>6 mm</td>
<td>Mean/total</td>
<td>68</td>
<td>120</td>
<td>103</td>
<td>95</td>
<td>17</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>±8.7</td>
<td>58.8</td>
<td>46.2</td>
<td>42.6</td>
<td>7.6</td>
<td>49.8</td>
<td></td>
</tr>
<tr>
<td>7 mm</td>
<td>Mean/total</td>
<td>66.1</td>
<td>97</td>
<td>21</td>
<td>60</td>
<td>13</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>±8.8</td>
<td>82.2</td>
<td>17.8</td>
<td>50.9</td>
<td>11.0</td>
<td>38.1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Mean/total</td>
<td>67.5</td>
<td>226</td>
<td>115</td>
<td>187</td>
<td>29</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>±8.8</td>
<td>66.3</td>
<td>33.7</td>
<td>54.4</td>
<td>8.5</td>
<td>36.7</td>
<td></td>
</tr>
</tbody>
</table>

AK, Above knee; BK, below knee; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Thrombosantin, Boehringer, Ingelheim, Germany) or anticoagulation treatment (marcumar) when clinically indicated.

Clinical follow-up. Clinical follow-up was performed as a prospective cohort evaluation. Patients were seen in the outpatients department at regular intervals (3 months, 6 months, 1 year, and annually thereafter) and when prompted by clinical deterioration. Over the entire observation period, only 11 patients were lost to follow-up, and 48 patients died. Given the fact that on the basis of a previous randomized trial the currently reported endothelialization program was defined as a routine-service for patients without saphenous veins, first-line patency assessment was based on significant impairment of claudication distances and walking impairment questionnaires allowing additional phone-in screening for patients who could not comply with routine follow-up appointments. If deteriorations were suspected, confirmation was based on ankle-brachial index (ABI) measurement. In case of deterioration of 15% or more or in patients with media-sclerosis angiography was performed. When Duplex sonography became a routine procedure soon after commencement of the program, it was adopted as the standard means of patency confirmation. If a significant graft stenosis or occlusion was confirmed an angiography was performed. Fifty-eight out of the initial 80 patients additionally underwent a planned control angiography after 1 year. Twenty-two patients refused consent.

Morphological investigations. Immediately after the grafts were taken out of the rotation device and prior to implantation, control specimens were taken for scanning electron-(SEM) and epifluorescence microscopy. Samples for the latter were stained with a vital dye combination (Live/Dead kit; Molecular Probes, Eugene, Ore). Specimen retrieved from eight patients undergoing graft revision were initially rinsed in PBS and then transferred into jars containing 4% formalin (0.1 mol/L in PBS/pH 7.2/4°C). After transfer to the laboratory, representative samples were cut off and further processed for (SEM) and histology. In a first step, SEM samples were transferred into 2% glutaraldehyde (0.05 M Cacodylate buffer/pH 7.2/4°C). After 48 hours of fixation, samples were dehydrated in graded ethanol, critical point dried (CDP 20; Balzers, Liechtenstein) and investigated with a Jeol JSM 5200 (Tokyo, Japan). Specimen for histology were further fixed in formalin and then embedded in paraffin. Three mm thick sections were either stained for light microscopy Hemotoxillin-Eosin (HE), Azan, Movat) or processed for immune-fluorescence microscopy (Nikon Eclipse 90i; Nikon, Tokyo, Japan). Antibodies used were directed against CD31 (Dako Cytomation M0823; monoclonal; Glostrup, Denmark), a-SM actin (Fitzgerald Industries, International Inc., Concord; Mass RDI-ACTINabm-A4; monoclonal), Ham 56 (Diagnostech; M0632; monoclonal; Glostrup, Denmark) and CD 68 (Dako Cytomation; M0814; clone KP1; monoclonal; Glostrup, Denmark), Von Willebrand Factor-FVIII (Dako Cytomation; A0082; polyclonal; Glostrup, Denmark).

Statistics. Statistical analysis of primary patencies was performed using the Kaplan-Meier survivorship function (Chicago, Ill). Patencies were compared by the log-rank test and Gehan’s Wilcoxon test. For group comparisons of culture data, Student unpaired t test was used. Preoperative fasting glucose-, cholesterol-, triglyceride (TG)-, lipoprotein A-, high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-levels were partitioned by follow-up gap. “Partitioning” was deployed using JMP, a statistical software package (version 6.0.3, Cary, NC). Recursively, partitions, in this case patients, based on a hypothetical relationship between patency and the various parameters.

<table>
<thead>
<tr>
<th>Table. Continued</th>
<th>Patients</th>
<th>IDDM</th>
<th>NIDDM</th>
<th>Glucose</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>HDL</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>On Warfarin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>33</td>
<td>23</td>
<td></td>
<td>123.32</td>
<td>218.39</td>
<td>209.79</td>
<td>52.82</td>
<td>124.87</td>
</tr>
<tr>
<td>17.0</td>
<td>21.6</td>
<td>15.0</td>
<td></td>
<td>72.56</td>
<td>42.80</td>
<td>141.24</td>
<td>17.93</td>
<td>27.45</td>
</tr>
<tr>
<td>45</td>
<td>45</td>
<td>25</td>
<td></td>
<td>113.64</td>
<td>209.53</td>
<td>196.48</td>
<td>53.46</td>
<td>119.44</td>
</tr>
<tr>
<td>29.0</td>
<td>29.0</td>
<td>16.1</td>
<td></td>
<td>58.68</td>
<td>55.19</td>
<td>128.00</td>
<td>14.26</td>
<td>36.93</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>3</td>
<td></td>
<td>106.11</td>
<td>221.75</td>
<td>209.06</td>
<td>51.13</td>
<td>115.37</td>
</tr>
<tr>
<td>72.7</td>
<td>24.2</td>
<td>9.1</td>
<td></td>
<td>53.27</td>
<td>87.03</td>
<td>148.66</td>
<td>19.46</td>
<td>66.38</td>
</tr>
<tr>
<td>66</td>
<td>47</td>
<td>36</td>
<td></td>
<td>117.85</td>
<td>221.79</td>
<td>219.79</td>
<td>53.62</td>
<td>120.63</td>
</tr>
<tr>
<td>36.6</td>
<td>24.0</td>
<td>18.4</td>
<td></td>
<td>68.23</td>
<td>56.82</td>
<td>151.81</td>
<td>15.49</td>
<td>36.36</td>
</tr>
<tr>
<td>29</td>
<td>39</td>
<td>15</td>
<td></td>
<td>116.65</td>
<td>203.33</td>
<td>173.89</td>
<td>51.83</td>
<td>124.33</td>
</tr>
<tr>
<td>25.9</td>
<td>34.8</td>
<td>13.4</td>
<td></td>
<td>58.89</td>
<td>44.51</td>
<td>90.10</td>
<td>16.47</td>
<td>35.15</td>
</tr>
<tr>
<td>95</td>
<td>86</td>
<td>51</td>
<td></td>
<td>117.44</td>
<td>215.66</td>
<td>204.83</td>
<td>52.79</td>
<td>122.31</td>
</tr>
<tr>
<td>27.9</td>
<td>25.2</td>
<td>15.0</td>
<td></td>
<td>65.07</td>
<td>53.74</td>
<td>136.37</td>
<td>15.92</td>
<td>35.74</td>
</tr>
</tbody>
</table>

IDDM, Insulin-dependent diabetes melitus; NIDDM, non insulin-dependent diabetes melitus.
improved from 0.53 (Patients at risk are shown in the Table). The ABI had confirmed all implanted grafts as noninfected.

Microbiology and the postoperative clinical course bacterial infection and replaced by a newly harvested contamination. One first-passage culture was found positive for grafts were regularly examined regarding microbial confluence, 12% of grafts showed pre-confluence, and only 1% displayed small nonendothelial-covered areas. Cultures and influence, 12% of grafts showed complete confluence, 12% of grafts showed pre-confluence, and only 1% displayed small nonendothelial-covered areas. Cultures and grafts were regularly examined regarding microbial contamination. One first-passage culture was found positive for bacterial infection and replaced by a newly harvested culture. Microbiology and the postoperative clinical course confirmed all implanted grafts as noninfected.

**Clinical follow-up.** The observation time ranged from 1 month to 14.2 years with a mean period of 9.6 years (Patients at risk are shown in the Table). The ABI had improved from 0.53 ± 0.18 preoperatively to 0.93 ± 0.31; 0.93 ± 0.30 and 0.87 ± 0.27 at 3 months, 6 months, and 12 months, respectively. It had significantly dropped to 0.39 ± 0.17 prior to intervention. The Kaplan-Meier survivorship function for femoropopliteal grafts showed an overall primary patency rate of 69% at 5 years and 61% at 10 years with a limb salvage rate of 94% and 89%. The secondary patency was 92% and 85% (Fig 2, a). Differences between stage I and stage II/III patients were not significant (Fig 2, b). Aboke knee grafts had a slightly worse patency rate than below knee grafts (68% vs 71% and 59% vs 64% at 5 and 10 years, respectively; N.S.) (Fig 3, a). For both anatomic positions differences between stage I and stage II/III patients were not significant (Fig 3, b and c). When 7 mm prostheses (n = 112) were compared with 6 mm ones (n = 196) there was a significantly better patency in the 7 mm group (78% vs 62% at 5 years; log rank test \( P = .023 \); Breslow test \( P = .017 \) (Fig 4). The distribution of run off between 6 mm and 7 mm graft was almost identical with only 6% more patients with a single run-off vessel in the 6 mm group. Comparisons between patient groups receiving 6 mm and 7 mm grafts showed no significant differences neither with regards to the number of run-off vessels nor to clinical staging. Since 7 mm grafts were only implanted from 1996 onwards the longest observation period for this graft diameter was 11.5 years. The overall patency for femorodistal reconstructions was 52% and 36% at 5 and 10 years with a limb salvage rate of 86% and 71%.

**Occlusions/critical stenoses.** Early occlusions (30 days postoperatively) accounted for 5% of all events, midterm occlusions (30 days to 3 years postimplantation) for 73% and long-term occlusions (>3 years) for 22%. Of all occlusions, 35% were thrombectomized; 29% were re-operated and received a new bypass graft (25% of which were in vitro endothelialized again); 18% had the graft lysed and in the remaining 18% of all occlusions no intervention was indicated.

**Angiographic findings.** Control angiographies after 1 year largely showed a widely open lumen with no narrowings or surface irregularities. In three out of 58 grafts moderate wall irregularities were found in the central two-thirds of the prosthesis. Moderate stenoses were further seen at the proximal anastomosis in two cases and at the distal anastomosis in one case. Even distal reconstructions showed largely pristine year one control angiographies (Fig 5, a). Minor anastomotic aneurysms were seen in two patients, one proximal and one distal. None of these changes required intervention.

**RESULTS**

**Endothelial cell cultures and graft lining.** Overall, only 2.5% of the 318 patients did not receive an endothelialized graft because of growth failure of endothelial cells. In the remaining patients primary cultures were ready for passage on day 9.1 ± 4.0 and seeding of approximately 18 million cells (18.1 ± 8.1 × 10^6) onto the grafts was possible on day 18.9 ± 4.5. After an additional post-seeding maturation period of 8.7 ± 2.2 days grafts were implanted 27.6 ± 7.8 days after vein excision. SEM and vital fluorescence controls of the endothelialization process showed a completely confluent endothelium in 78% of freshly seeded grafts (Fig 1, a). Sixteen percent of the remaining grafts were pre-confluent lined (Fig 1, b) and only 6% had minor endothelium-free areas (Fig 1, c). At the time of implantation, 87% of grafts showed complete confluence, 12% of grafts showed pre-confluence, and only 1% displayed small nonendothelial-covered areas. Cultures and grafts were regularly examined regarding microbial contamination. One first-passage culture was found positive for bacterial infection and replaced by a newly harvested culture. Microbiology and the postoperative clinical course confirmed all implanted grafts as noninfected.

**Occlusions/critical stenoses.** Early occlusions (30 days postoperatively) accounted for 5% of all events, midterm occlusions (30 days to 3 years postimplantation) for 73% and long-term occlusions (>3 years) for 22%. Of all occlusions, 35% were thrombectomized; 29% were re-operated and received a new bypass graft (25% of which were in vitro endothelialized again); 18% had the graft lysed and in the remaining 18% of all occlusions no intervention was indicated.

**Angiographic findings.** Control angiographies after 1 year largely showed a widely open lumen with no narrowings or surface irregularities. In three out of 58 grafts moderate wall irregularities were found in the central two-thirds of the prosthesis. Moderate stenoses were further seen at the proximal anastomosis in two cases and at the distal anastomosis in one case. Even distal reconstructions showed largely pristine year one control angiographies (Fig 5, a). Minor anastomotic aneurysms were seen in two patients, one proximal and one distal. None of these changes required intervention.
Angiographies done for deteriorating claudication distances, ABI and Duplex sonographic flow assessment showed complete or sub-total thrombotic graft occlusion in 69%. Stenotic changes that required an intervention were found in 31% of all events, of which 17% were proximal, 33% in the midsegment (Fig 5, a) and 50% in the distal section of the grafts.

Morphological findings in failed grafts. Specimens were obtained from eight patients requiring graft revision 38.9 ± 17.8 months after implantation (ranging from 7 to 63 months). Seven out of eight revisions were elective procedures 5.3 ± 4.3 months after significant in-graft stenoses had been diagnosed by ultrasound or angiography. Only one of the stenoses was at the distal anastomosis; the remainder occurred within the mid two-thirds of the grafts. Histologically, all samples showed eccentric intralumenal tissue formations largely resembling the scar tissue (pannus) of burnt-out anastomotic intimal hyperplasia (Fig 6, a and b). This pannus-like tissue was regularly covered by a confluent CD 31/F VIII-positive endothelial layer. The remaining circumference of the cross sections showed three distinctive tissue formations in all explants: (1) slightly wavy layers of largely acellular material directly attached to the ePTFE surface and covered by a confluent endothelium; (2) cell-rich formations of well-aligned α-actin-positive cells (Fig 7, a) often separated form the confluent endothelium by a distinct, wavy elastin membrane (Fig 7, b); and (3) stretches of densely-packed inflammatory cells resting on the ePTFE surface and being directly covered by a loose sheet of endothelium (Figs 8 and 9). While foreign body giant cells (FBGC) were regularly present in these inflammatory areas (Fig 8, a and b; Fig 9), macrophages (Fig 8, d) and lymphocytes (Fig 8, e) were alternately the predominant cell type. Sometimes, dense front-lines of macrophage-debris infiltrated the surface layer of the ePTFE (Fig 8, d). Granulocytes were completely absent. In two of the grafts large thrombi were attached to these hyper-inflammatory areas (Fig 9). In three of the patients large pockets of foam cells were found in various locations between the pannus-like tissue and the ePTFE surface (Fig 6, c and d).

In general, SEM confirmed the presence of an endothelium on all specimens. Occasionally, some areas were patchy while others showed overgrowth onto the often spongy fibrin formations extending from adjacent thrombi.

Risk factor assessment. There was no significant difference in patency between males and females. Partitioning of risk factor values by follow-up gap suggested cut-points for the cohort of patients receiving endothelialized grafts of 203 mg% for glucose; 223 mg/dL for cholesterol; 133 mg/dL for triglycerides; 39 mg/dL for Lipoprotein A; 62 mg/dL for HDLC and 144 mg/dL for LDLC. Freedom from occlusion between the two sub-groups was only significant for triglycerides.
Thirty years after its conception an idea could be vindicated that was pioneered by vascular surgeons and eventually led to a development later termed “tissue engineering”. Unfortunately, its early failure to translate into a clinical benefit had also made vascular graft endothelialization the antithesis of promises in tissue engineering. After a quarter of a century of successive improvements, however, the endothelialization of vascular grafts had developed from an early prototype of tissue engineering into a clinically relevant and safe procedure that could be routinely offered to a sizable cohort of patients in a nontertiary institution.

By providing convincing clinical mid- to long-term results in femoropopliteal bypass patients who had no autologous conduit available, we previously showed that a cell culture-based approach may lead to a significant clinical benefit. By carrying the method over into a routine procedure at a community hospital, we could counter the perception that in vitro endothelialization required the sophisticated infrastructure of a tertiary institution. Similarly, the successful implantation of a confluent endothelialized graft into 97.5% of patients from whom cells were cultured as well as the absence of graft infection confirmed the procedure as a reliable routine therapy. By embedding it into a large cohort of consecutive patients, we could demonstrate that in spite of the culture-related delay, two-thirds of infrainguinal bypass patients who have no suitable saphenous vein available could profit from the method.

Given the fact that our group has been implanting almost 400 in vitro endothelialized ePTFE grafts over a period of 20 years, procedure- and design-related changes evolved over time. One of these was the continuation of the program as a routine-service for all patients without a suitable saphenous vein from 1993 onwards after the initial randomized study in 50 patients showed significantly better patencies with 74% at 7 years in the endothelialized ePTFE group. Although this step deprived the current cohort of a nonendothelialized control group, 40 years of clinical data on ePTFE- and saphenous vein grafts provide well-established baselines. Particularly in view of the fact that the patients represented in our cohort were all lacking a suitable saphenous vein, patency rates resembling or surpassing those of saphenous vein grafts need to be seen against the particularly low ePTFE results in this subgroup. The consistent proof of an endothelium in explanted samples obtained between 7 and 63 months after implantation as well as a trend towards better patencies in the below knee group further support the view that the endothelialized grafts behave more like vein grafts than conventional ePTFE grafts. While this trend had already been apparent when we reported on the initial 136 patients of the present cohort, an observation period twice as long and by now a total of 310 patients who received an endothelialized ePTFE graft affirmed the previous patency rates. Although the first-line determination of long-term patency was primarily based on clinical assessment, its confirmation

Fig 5. (a) Stenosed mid-graft segment of an in-vitro endothelialized ePTFE graft. (b) One-year follow-up angiography of an endothelialized 6 mm graft used in a distal reconstruction. Smooth surface of the endothelialized prosthesis and no distal anastomotic narrowing. An additional bridge-graft (B) reconstruction had been performed between the posterior tibial and the peroneal artery with the endothelialized graft inserted near the proximal bridge anastomosis.

DISCUSSION

Thirty years after its conception an idea could be vindicated that was pioneered by vascular surgeons and eventually led to a development later termed “tissue engineering”. Unfortunately, its early failure to translate into a clinical benefit had also made vascular graft endothelialization the antithesis of promises in tissue engineering. After a quarter of a century of successive improvements, however, the endothelialization of vascular grafts had developed from an early prototype of tissue engineering into a clinically relevant and safe procedure that could be routinely offered to a sizable cohort of patients in a nontertiary institution.

By providing convincing clinical mid- to long-term results in femoropopliteal bypass patients who had no autologous conduit available, we previously showed that a cell culture-based approach may lead to a significant clinical benefit. By carrying the method over into a routine procedure at a community hospital, we could counter the perception that in vitro endothelialization required the sophisticated infrastructure of a tertiary institution. Similarly, the successful implantation of a confluent endothelialized graft into 97.5% of patients from whom cells were cultured as well as the absence of graft infection confirmed the procedure as a reliable routine therapy. By embedding it into a large cohort of consecutive patients, we could demonstrate that in spite of the culture-related delay, two-thirds of infrainguinal bypass patients who have no suitable saphenous vein available could profit from the method.

Given the fact that our group has been implanting almost 400 in vitro endothelialized ePTFE grafts over a period of 20 years, procedure- and design-related changes evolved over time. One of these was the continuation of the program as a routine-service for all patients without a suitable saphenous vein from 1993 onwards after the initial randomized study in 50 patients showed significantly better patencies with 74% at 7 years in the endothelialized ePTFE group. Although this step deprived the current cohort of a nonendothelialized control group, 40 years of clinical data on ePTFE- and saphenous vein grafts provide well-established baselines. Particularly in view of the fact that the patients represented in our cohort were all lacking a suitable saphenous vein, patency rates resembling or surpassing those of saphenous vein grafts need to be seen against the particularly low ePTFE results in this subgroup. The consistent proof of an endothelium in explanted samples obtained between 7 and 63 months after implantation as well as a trend towards better patencies in the below knee group further support the view that the endothelialized grafts behave more like vein grafts than conventional ePTFE grafts. While this trend had already been apparent when we reported on the initial 136 patients of the present cohort, an observation period twice as long and by now a total of 310 patients who received an endothelialized ePTFE graft affirmed the previous patency rates. Although the first-line determination of long-term patency was primarily based on clinical assessment, its confirmation

Fig 5. (a) Stenosed mid-graft segment of an in-vitro endothelialized ePTFE graft. (b) One-year follow-up angiography of an endothelialized 6 mm graft used in a distal reconstruction. Smooth surface of the endothelialized prosthesis and no distal anastomotic narrowing. An additional bridge-graft (B) reconstruction had been performed between the posterior tibial and the peroneal artery with the endothelialized graft inserted near the proximal bridge anastomosis.
by duplex sonography provided a high degree of reliability. Since the main purpose of the study was to demonstrate that in vitro endothelialization may be provided as a routine service at the level of a community hospital, open-ended sophisticated follow-up procedures in asymptomatic patients exceeding the initial years would have been unfeasible. Nevertheless, a recent study\(^8\) on the relationship between quantitative and qualitative measures of symptomatic peripheral arterial disease found a significant correlation between the two, thus confirming walking impairment questionnaires as a reliable method of first-line patency screening.

One other development over time was the introduction of 7 mm prostheses for larger popliteal target vessels in year four. Although the significantly better patency rate of 7 mm grafts may well be due to a better run-off, a 5-year patency of 78% in the endothelialized 7 mm group is still remarkable particularly in a situation where the 6 and 7 mm groups neither differed with regards to clinical staging nor to the number of patent crural run-off arteries. A further inhomogeneity over time resulted from the developments associated with the human immunodeficiency virus (HIV) pandemic and the emergence of lipid-lowering drugs in the 1990s. After previous studies established a link between growth-failure of endothelial cells as well as patency of endothelialized grafts on the one hand and preoperative lipid levels on the other, growth-failure of primary endothelial cultures could be dramatically reduced from 27%\(^9\) to 5%\(^20\) by the timely exchange of the patients own serum with low-lipid pool-serum. With the emerging need of polymerase chain reaction-analyses for the exclusion of HIV infection and the availability of lipid-lowering drugs serum exchange was abandoned at an early stage and lipid-control became routine leading to an almost complete absence of growth failure. Since most patients were admitted when they were already on lipid-lowering drugs, our observation of a continual correlation of higher preoperative triglyceride levels with lower patencies of the endothelialized grafts needs to be seen in perspective. Nevertheless, since statins do not lower TGs by more than 10% to 15% and higher TG are associated with more remnants of atherogenic small dense LDL and lower HDLC the correlation still holds predictive value. This is particularly important in view of the distinct atherogenic lesions found in a

---

Fig 6. Midgraft segments explanted at the time of re-operation for graft failure 41 months (a) and (c) and 63 months (b) and (d) after implantation of an in vitro endothelialized ePTFE graft. Both specimens contained other areas of more significant stenoses but the displayed prestenotic regions were packed with large islands of foam cells (F) (c) and (d). Typically, the foam cells were wedged underneath pannus-like cell-poor tissue that occasionally showed stretches of complete acellularity (A) (c). Masson's Trichrome Stain; “stitched” images to represent objective magnifications of ×0.5 (a) and (b) and ×10 (c) and (d).
significant proportion of the samples we obtained from failed endothelialized grafts.

This leads to the main question of how endothelialized ePTFE grafts fail. At first glance, thrombosis seems to be prevalent occurring primarily between day 30 and 3 years nurturing the suspicion that the endothelium may have disappeared. Histologic analysis of failed midgraft segments, however, revealed that highly inflammatory subendothelial areas together with hyperplastic narrowings may account for these occlusions rather than the absence of an endothelium. Although one needs to keep in mind that midgraft specimens were only obtained from eight patients, they all stem from failed grafts. The most significant finding was that subendothelial connective tissue cells were present in abundance in midgraft regions that otherwise hardly ever show tissue formations in non-endothelialized ePTFE grafts.

The source of the often smooth muscle-like cells could either be contamination during culture, something which would have been noticed in the course of 19 days of mass culture, homing of circulating progenitor cells enabled through the presence of an endothelium or transdifferentiated endothelial cells. The often eccentrically narrowing formations of a pannus-like tissue, whose potential precursors may be the mild luminal irregularities visible on 5% of year one angiographies in the present cohort and described by others,11 presented a

Fig 7. Different area of the same specimen as in Fig 6a and c of an endothelialized graft 41 months after implantation. Relatively extended areas were typically found in most explants where a confluent endothelium was resting on a layer of well-aligned α-SMC actin-positive cells (a) (Immunofluorescence α-SMC actin/CD31; Dapi nuclear counter stain/CD 31; ×10 objective) A delicate intima was demarcated from the actin positive cells by a well-defined elastic membrane. Orcein stain; ×10 and ×20 objectives.

Fig 8. Areas of confluent endothelium (*) lying in direct proximity to densely packed inflammatory cells, which were directly resting on the ePTFE surface without any connective tissue cells. Explant samples of four different patients 27 months (a), 14 months (b), 29 months (c), and 63 months (d) after implantation. Foreign body giant cells (*) were regularly present, either directly attached to the ePTFE (a) or tucked underneath the endothelium (b). Some areas consisted almost exclusively of lymphocytes (c) while others were dominated by macrophages (d). Occasionally, macrophage debris (**) was densely packed into the surface layer of the ePTFE (d). Double-immunofluorescence Ham56/CD68 and CD31; Dapi nuclear stain; ×10 (a), (c), and (d), and ×40 (b) objectives.
mixed picture between the cell-poor fibrous cap of an arteriosclerotic lesion and its large accumulations of foam cells in the depth. Most peculiar, however, were those stretches where a thin layer of endothelium was directly attached to a densely packed layer of inflammatory cells wedged between the endothelium and the ePTFE surface. These areas were associated with sometimes large surface thrombi confirming the observation of the 1980s that inflammation turns an endothelium into a procoagulatory entity. The proinflammatory role of lipids in this process can just be speculated.

In summary, we have shown in a cohort of 341 infrainguinal in vitro endothelialized PTFE grafts that a largely confluent autologous endothelium can be safely provided on ePTFE prostheses for 97.5% of elective patients within less than 4 weeks and without the occurrence of infections. Since only one-third of the overall 465 patients were unable to wait for the completion of the procedure due to acute clinical symptoms, autologous in vitro endothelialization can be seen as a potential therapy for two-thirds of the patients in need of an infrapopliteal bypass grafts who have no suitable saphenous vein available. Long-term patencies resembling vein grafts together with the fact that the procedure has been uninterruptedly provided as a routine therapy at a nontertiary hospital for 15 years may help to overcome the perception that complexity outweighs the benefit.

AUTHOR CONTRIBUTIONS

Conception and design: PZ
Analysis and interpretation: PZ, NH, MG, AF, AS, DB, MG
Data collection: JM
Writing the article: PZ

REFERENCES

DISCUSSION

Dr G. Patrick Clagett (Dallas, Tex). Have you used platelet-imaging studies to document the creation of a nonthrombogenic surface and, thereby, prove indirectly that there are viable endothelial cells on the prosthetic surface?

Dr Peter Zilla. We have done a preclinical study in primates in the mid-’80s. What it clearly demonstrated was a nonthrombogenic surface. However, in the beginning, you see quite a lot of inflammatory cells on top of the in vitro-lined endothelium, which after 4 weeks have completely disappeared.

Dr Rajabrata Sarkar (San Francisco, Calif). Many groups have tried for years to get endothelium to grow on PTFE [polytetrafluoroethylene] grafts. And now we know that stem cells are always attracted to sites of vascular inflammation. And my question for you is: Do you think that the cells that you find at explantation are actually the endothelial cells that you seeded, or have you just, with the fibrin glue, created a better landing site for these circulating endothelial-like stem cells?

Dr Zilla. This is a very good point. I would go one step further and expand it beyond the endothelial cells. The fact that we found actin-positive cells in the subendothelial layers and, in some areas, even tissue that resembles a vascular wall, makes one wonder how these cells got there. So far, we could not answer whether the originally transplanted endothelial cells transdifferentiated into actin-positive cells or whether the graft surfaces were homing sites for circulating cells.

Dr Sarkar. And my follow-up question is: How do you confirm that these are actually endothelial cells? Because exposed smooth muscle cells, when exposed to laminar flow, will line up in an endothelial cell-like monolayer.

Dr Zilla. I showed a series of immunofluorescence pictures. We didn’t only use factor VIII but also CD31 and transmission electron microscopy. The surface cells were all very clearly endothelial cells.

Dr Alexander Clowes (Seattle, Wash). I am intrigued by your morphology. Have you had the opportunity to do longitudinal studies using duplex scanning or MR [magnetic resonance] to find out the time course of how and where these lesions form?

Dr Zilla. It would have been great had we also done such a clinical study in a tertiary hospital, but this study was done in a community hospital with the purpose of demonstrating that the method can really be done everywhere as a clinical routine service without academic sophistication. Therefore, the answer is no, we don’t have this information, but the fact that the 1-year follow-up hardly showed any surface irregularities indicates that the process is slow and scarce.

Dr Kai Balzer (Dusseldorf, Germany). Three questions. First, the hope to make the layer of endothelium to inhibit myointimal hyperplasia. The pictures you showed kind of questioned that from the few specimens you obtained. The second question, have you ever tried—because from our experience, endothelial cells that you gain from veins sometimes lose the possibility to proliferate after several cell cycles—have you ever tried endothelial progenitor cells from the bone marrow? And the third question would be, have you ever tried labeling your cells that maybe even after 1 year you could see if those cells that you implanted were the ones still on the graft.

Dr Zilla. Let me start with the first question. The fact that in some areas we end up with a double layer where the actin-positive subendothelial cells show signs of a contractile phenotype indicates that the cells on the graft surface may attempt to emulate something physiological. Why other areas have an endothelium lying on naked blank PTFE after 63 months with inflammatory cells stuffed in between, I can’t explain.

With regards to labeling: Remember, Manfred Deutsch and I are veterans in that field, so even the last leg of the academic part of the study was performed more than 15 years ago, when methods such as adeno-associated viruses to deliver green fluorescent protein were not available yet. But I would love to see a follow-up on that question done by younger colleagues.

Dr Balzer. And progenitor cells?

Dr Zilla. The same applies here: based on the encouragement of the long-term clinical results, a new round of academic interest seems justified.