Eur J Vasc Endovasc Surg **11**, 105–111 (1996)

Re-endothelialisation in Autogenous Vein Grafts*

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Objectives: *To clarify the course of re-endothelialisation (Re-E) in an entire graft and to establish the effect of immersion media for the preservation of endothelial cells.*
Methods: *Autogenous femoral veins of dogs were immersed in heparinised saline solution (n = 18) or heparinized autogenous blood (n = 18). After immersion, the grafts were implanted into bilateral femoral artery, and were retrieved 1 day to 4–8 weeks after implantation.*
Results: *For the grafts immersed in the heparinised saline solution, the values for % area of endothelial cell coverage before implantation, and at 1 day, 1 week, and 4 weeks after implantation were 44.9%, 6.2%, 14.5%, and 81.3%, respectively. For the grafts immersed in heparinised autogenous blood, the values were 73.5%, 20.6%, 79.2% and 95.5%, respectively. However, such relatively rapid speed of Re-E slowed down considerably after 1 week following implantation in this group.*
Conclusions: *The use of heparinized autogenous blood is strongly recommended as a preparation media for autogenous vein grafts. Almost all of the endothelial cells fall away in the earlier period after implantation and regenerate multifocally and irregularly. Re-E is incomplete even at 8 weeks after surgery, and we suggest that the area of incomplete Re-E may develop into intimal hyperplasia.*

Key Words: Re-endothelialisation; Endothelial cell; Vein graft; Intimal hyperplasia; Silver staining method.

Introduction

In clinical studies of arterial reconstructions using autogenous vein graft, progressive intimal hyperplasia is the main cause of late vein graft failure. However, the mechanism of intimal hyperplasia is not clearly understood. Endothelial cell injury during preparation of grafts and vein graft quality are thought to be important factors in the development of intimal hyperplasia.[1–4] Scanning electron microscopy (SEM) of the luminal surface of the stenotic sites indicates that incomplete re-endothelialisation (Re-E) occurs. Since complete Re-E occurs at the non-stenotic site of the same grafts, we considered that intimal hyperplasia was clearly related to the delayed Re-E.

The length of time taken for Re-E depends on the extent of damage to the vein graft at operation. In previous studies, the process, speed and completion of Re-E have not been demonstrated clearly, since the investigations of the graft using SEM has not been available for the detailed study of Re-E in an entire graft after implantation.[7–11]

In this study, we have used a silver staining method to measure the area of endothelial cell coverage of an entire graft after implantation, and to compare the effects of heparinised autogenous blood and heparinised saline solution immersion on the preservation of the endothelial cell.

Material and Methods

Thirty-six adult mongrel dogs of either sex (weighting 10–15kg) were anesthetized with phenobarbital. Six centimetre lengths of both femoral veins were harvested using a no touch technique, distended by gentle manual pressure and immersed in heparinized (10U/ml) saline solution ($n = 18$), or heparinized (250U/ml) autogenous blood ($n = 18$) for 1 h at room temperature (20°C). The femoral vein then was reversed and implanted into the ipsilateral femoral artery with end-to-end anastomoses using 7-0 polypropylene. After implantation, the grafts were removed at 2 h, at 1, 2, 3, 5 and 7 days, and at 2, 3, 4

*Presented at the 36th Annual World Congress of the International College of Angiology, New York, July 1994.

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and 8 weeks. The specimens were opened longitudinally and fixed to a board immediately. Physical handling was minimised before staining. They were immersed in 0.3% silver nitrate solution for 2 min and fixative for 1 min under an ultraviolet lamp (UVL-56, UVP) with a longwave (365nm) at a distance of 15cm. Thereafter, the silver-stained preparations were rinsed gently with 20ml of 5% dextrose, and fixed with 10% formalin or 1% glutaraldehyde in phosphate-buffered saline solution for 1 h. The boundaries of individual endothelial cell coverage were traced from light microscope (LM). Using a computer image analyser (Nachet NS-150, image processor), the following was calculated: %area of endothelial cell coverage = area of endothelial cell coverage / total area of the luminal surface of the graft × 100. The silver staining method was not specific for identifying endothelial cells, so it was combined with a more specific method. SEM at higher magnification and staining for Factor VIII-related antigen were performed in all preparations to confirm the presence of endothelial cells. Statistical analysis of the data was performed by Student *t*-test for paired samples.

Results

Morphometric analysis

LM examination revealed a brown area and light-brown area intermingled in the luminal surface of the en-face silver-stained graft. Upon detailed examination of the brown area of the graft, the brown endothelial cell junctions were traced with silver lines, producing a characteristic "cobblestone appearance", while the shapes and sizes of the endothelial cells exhibited various patterns (Fig. 1A). In this area, SEM clearly demonstrated the presence of the spindle-shaped endothelial cells (Fig. 1B). Detailed investiga-

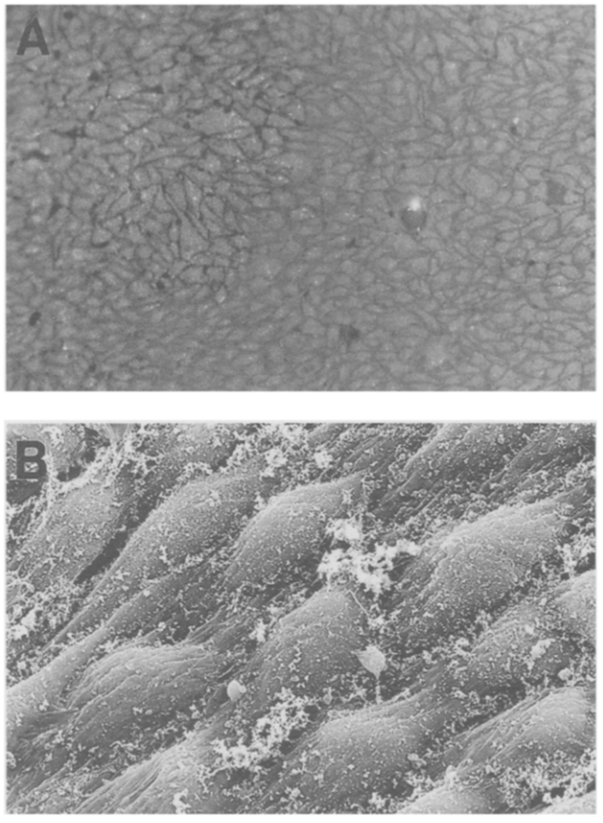

Fig. 1. A graft 5 days after implantation. (A) Light micrograph with silver staining demonstrates the endothelial silver lines (original magnification × 128). Note the "cobblestone appearance" of stained intercellular junction. (B) SEM clearly demonstrates the intact endothelial cells with distinct junction (original magnification × 1000). (Reproduced here at 80%).

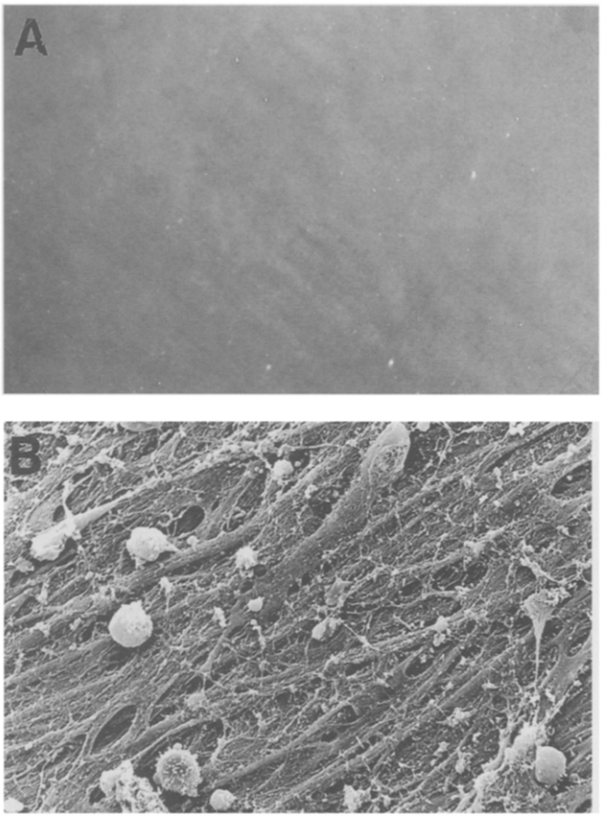

Fig. 2. A graft 5 days after implantation. (A) Light micrograph with silver staining did not demonstrate the "cobblestone appearance" (original magnification × 50). (B) SEM demonstrates the loss of endothelial cells and the exposure of subendothelial collagen fibres (original magnification × 500). (Reproduced here at 80%).

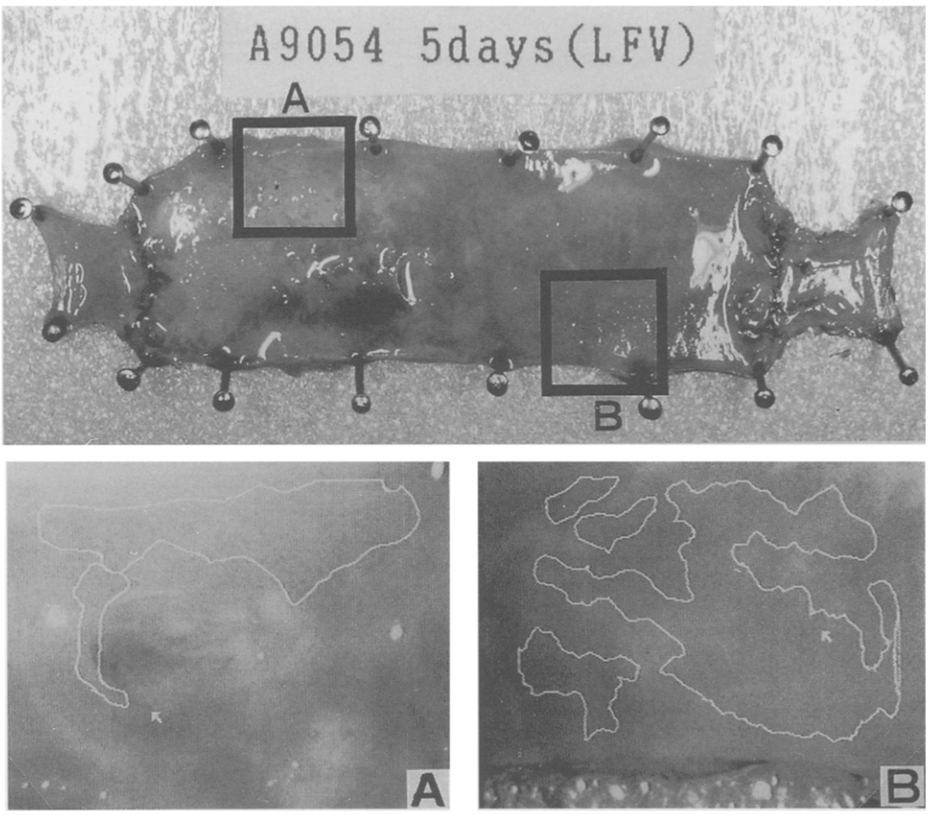

Fig. 3. Area of endothelial cell coverage on a graft 5 days after implantation. Light micrographic findings demonstrated the traced area showing a "cobblestone appearance" at the squared area A and B in the luminal surface of the graft (original magnification × 15).

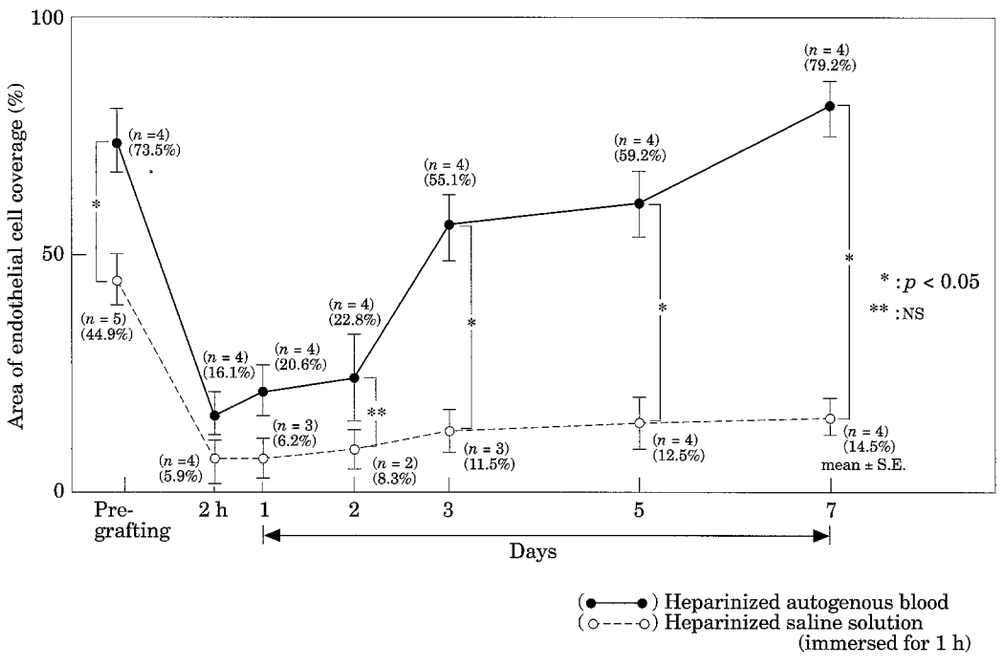

Fig. 4. %Area of endothelial cell coverage during the period from 2h to 7 days after implantation.

tion of the light-brown area was also performed at this time, but LM did not reveal the silver lines of "cobblestone appearance" and the luminal surface of the graft was unstained (Fig. 2A). SEM in this area demonstrated the loss of endothelial cells, and the exposure of collagen fibres in the subendothelial layer and internal elastic lamina (Fig. 2B). On the luminal surface of the graft at the early period after implantation, a few endothelial cells remained around the venous valve, the orifice of the branch, and the free luminal wall. After that, endothelial cells regenerated multifocally and irregularly on the luminal surface of the graft (Fig. 3).

Endothelial cell coverage assessment

After 1 h immersion prior to implantation, the endothelial cells in the entire graft immersed in heparinised autogenous blood and in heparinised saline solution covered an area of 73.5% and 44.3%, respectively. For the grafts immersed in heparinised autogenous blood, the values for %area of endothelial cell coverage at 2 h, and at 1, 2, 3, 5 and 7 days after implantation were 16.1%, 20.6%, 22.8%, 55.1%, 59.2% and 79.2%, respectively. For the grafts immersed in heparinised saline solution immersion, the values were 5.9%, 6.2%, 8.3%, 11.5%, 12.5% and 14.5%, respectively (Fig. 4). Almost all of the endothelial cells

Table 1. The effect of immersion media on % area/day*

Postoperative periods	% area/day (%/day) HB	HS
1–7 days	9.76±0.32**	1.38±0.18**
1–4 weeks	0.78±0.21**	3.18±0.25**

HB=Heparinised Autogenous Blood; HS=Heparinised Saline Solution.

* $\%\text{Area/day} = \frac{\text{Area of Endothelial Cell Coverage}}{\text{Total area of Vein Graft}} \times 100$

** $p<0.05$.

fell away from the luminal surface of the graft in the earlier period after implantation and regenerated gradually (Table 1). At 2, 3 and 4 weeks after implantation, the values for %area of endothelial cell coverage were 86.8%, 93.5% and 95.5%, respectively, for the grafts immersed in heparinised autogenous blood, and the values were 35.7%, 47.0% and 81.3%, respectively, for grafts immersed in heparinised saline solution. The %area of endothelial cell coverage was 93.3% at 8 weeks after implantation (Fig. 5) i.e. Re-E was still incomplete even at 8 weeks after surgery (Fig. 6).

Discussion

The main cause of late graft failure is progressive

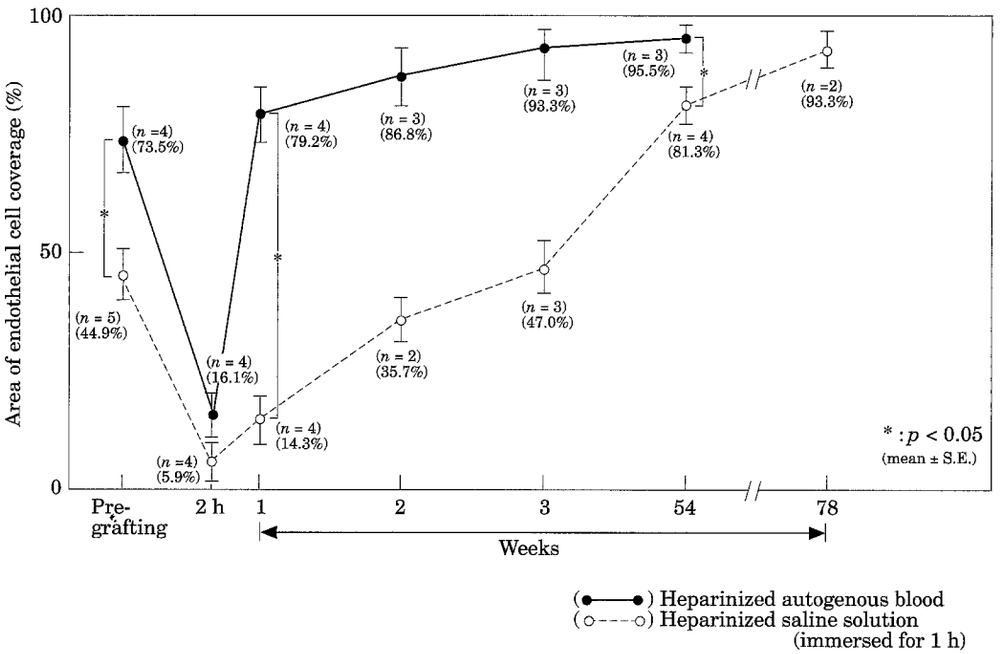

Fig. 5. %Area of endothelial cell coverage during the period from 1 week to 8 weeks after implantation.

intimal hyperplasia, and the incidence of this complication is concentrated within the first 2 years after implantation, as has been previously reported.[12,13] However, the mechanism and prevention of intimal hyperplasia has not been demonstrated clearly. Even now, the process of Re-E and the effect of immersion media have not been fully clarified. When an autogenous vein graft is implanted an artery, endothelial cell loss occurs before implantation due to the physical and chemical injuries that occur in the preparation and immersion.[8,14] Most of the endothelial cells fall away rapidly from the luminal surface of the implanted graft following the exposure to the arterial environment and it's shear stress.[4,5] Re-E of the graft commences gradually, and smooth muscle cells start migrating from the media across the intimal elastic lamina and continue to proliferate to form intimal hyperplasia. As soon as Re-E is completed, the growth of smooth muscle cells appears to stop. Thus, intimal hyperplasia is a time-limited process of the autogenous vein graft.[6,8,15,16] Nevertheless, in up to 15% of the area of the implanted graft, intimal hyperplasia does not stop, and continues to proliferate.[13] Vascular smooth muscle cell proliferation and intimal hyperplasia are a common response to endothelial injury.[17,18] The initial stimulus for this proliferative response is thought to be platelet-derived growth factor released from platelets attached to the site of injury.[19] The formation of intimal hyperplasia after vascular injury depends on smooth muscle cell proliferation, migration, and synthesis, and is regulated by positive and negative factors from the blood, leukocytes, and the vascular wall themselves.[15]

There have been many previous reports regarding the Re-E of autogenous vein grafts in dogs. These studies report conflicting results as to the point of completion of Re-E; that is, 10 days,[20] 2 weeks,[21] 3 weeks,[5,22] 6 weeks,[23,24] 10 weeks[25] and 12 weeks[26] after implantation (Table 2). These conclusions were varied because most investigators did not study the entire graft, but only a few local areas of the luminal surface using a SEM. However, using the silver staining method, which provided a broad overview for evaluation of the flow surface, we could trace the area showing "cobblestone appearance" in each graft, and measured the area of endothelial cell coverage in the entire graft using a computer image analyser.[27,28]

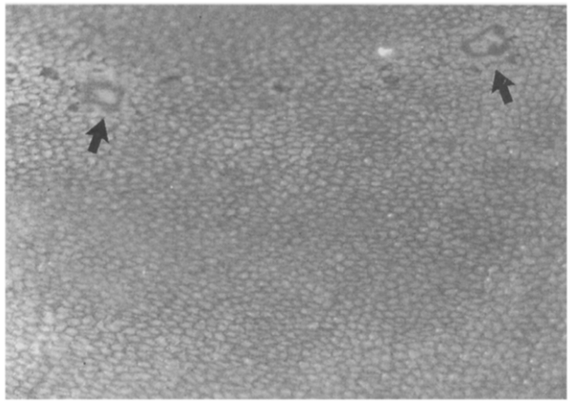

Fig. 6. A graft 8 weeks after implantation (immersed in heparinised saline solution). Note the existence of small areas of incomplete Re-E (arrows; original magnification × 128). (Reproduced here at 60%).

Table 2. Re-endothelialisation in autogenous vein grafts (Experimental studies)

Author	Completion of Re-E	Immersion media	Animals
Adcock (1987)	10 days	HB	Dog
Cambria (1985)	2 weeks	LR	Dog
Reichle (1973)	3 weeks	S	Dog
Horio (1991)	3 weeks	S	Dog
Bush (1986)	4–6 weeks	HB, HS	Dog
Hoover (1988)	6 weeks	HB, HS	Dog
Quist (1992)	10 weeks	S	Dog
Wyatt (1966)	12 weeks	S	Dog

HB=Heparinised whole blood; HS=Heparinised saline solution; S=Saline solution; LR=Lactated Ringer's solution.

The silver staining method demonstrated that endothelial cells regenerated multifocally and irregularly, and this fact suggested that local investigation of the graft using only SEM was not useful for the detailed study of Re-E in an entire graft. Using this silver staining method, silver granules were observed to be localised along the intercellular junctions, and also tended to pool in the basement membrane beneath each junction.[29] The endothelial cells were outlined with silver lines and revealed as a characteristic "cobblestone appearance." Since endothelial cells did not always exist in the brown stained area of the luminal surface, it was necessary to confirm the presence of silver lines and "cobblestone appearance" to confirm the existence of endothelial cells.[27]

There have been many previous reports about the effects of immersion media on autogenous vein grafts.[30–33] It is well known that the integrity of venous endothelial cells during graft preparation is strongly influenced by temperature, distention pressure, solution composition, osmotic pressure, and utilisation of pharmacologic agents.[20,34] We compared the maintenance of endothelial cells by heparinised autogenous blood and by heparinised saline solution. The present study established that heparinised autogenous blood was superior to heparinised saline solution for the preservation of endothelial cells. This

is probably because the heparinised autogenous blood has more physiological properties than heparinised saline solution in terms of buffer function, oxygen transportation, oncotic pressure and optimal pH.[34,35] Previous reports have also shown that blood storage preserves the integrity of the endothelial monolayer better than saline solution.[24,30] The effect of heparinised autogenous blood as the optimal preparation media has also been confirmed by investigating prostacyclin production and biochemical function of the endothelial cells.

In conclusion, the use of heparinised autogenous blood is strongly recommended as a preparation media for autogenous vein grafts. Endothelial cells regenerate multifocally and irregularly on the luminal surface of the vein graft. Re-E is not complete even at 8 weeks after surgery. We suggest that these areas of incomplete Re-E may develop into intimal hyperplasia. However, further detailed studies are required to establish whether vein graft stenosis due to intimal hyperplasia is directly related to areas of incomplete Re-E.

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Accepted 15 May 1995