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The rate of re-endothelialization correlates inversely with the degree of the following intimal thickening in vein grafts

Electron microscopic and immunohistochemical studies

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Summary. The distribution of re-endothelialization and the development of intimal thickening were investigated electron microscopically and immunohistochemically using saphenous vein grafts implanted into the femoral artery in dogs. Animals were divided into two groups according to a difference of preparation of the venous grafts before implantation: group 1 without storage, and group 2 with storage for 1 h in 0.9% sodium chloride solution containing papaverine and heparin. In group 2 endothelial cells were almost totally denuded at 1 day, while endothelial islands were always left on the back of valves. In group 1 small islands of surviving endothelial cells were occasionally observed away from the valves. By 1 week, the re-endothelialization of group 1 and group 2 extended to $71 \pm 14.7\%$ and $47 \pm 9\%$ (mean $\pm$ SD) of the total luminal surface area, respectively, and spread mainly from the valves but not from the adjacent artery. Immunohistochemical study using anti-dog fibrinogen IgG suggested that increased permeability of the graft wall continued for one month, in spite of the endothelial covering. Intimal thickening was most pronounced at 1 month, especially near the anastomosis, but was less in the middle of the grafts. These results indicate that intimal thickening of the venous grafts is a response to injury which intimately depends on the rate of re-endothelialization.

Key words: Saphenous vein – Endothelium – Smooth muscle cells – Fibrinogen

Introduction

Saphenous vein aortocoronary bypass grafts may undergo a variety of morphological changes that lead to both early occlusion by thrombosis and late stenosis by intimal thickening. Interactions between platelets and the blood vessel wall have been implicated in venous grafts occlusion (Brown et al. 1985; Dewanjee et al. 1984; Fuster and Chesebro 1986; Josa et al. 1981) as well as in the pathogenesis of atherosclerosis (Clowes and Karnowsky 1977; Friedman et al. 1977; Fuster and Chesebro 1985; Reidy 1986). Haudenschild and Schwartz (1979) have showed that there was less intimal thickening in the areas rapidly repopulated by endothelium in rat aorta, when compared with that in the areas where endothelial repopulation was slow. This finding suggests that there is a critical duration and range of endothelial loss, permitting smooth muscle cell migration and proliferation through the action of several effectors, including platelet derived growth factor (PDGF). Vein grafts exhibit morphological alterations of endothelial cells even before implantation, but the majority of the cells keep an attachment to the subendothelial tissue (Fuchs et al. 1978; Ramos et al. 1976; Roberts et al. 1984; Sottiurai et al. 1983). After implantation into the arterial circulation, endothelial cells subsequently undergo further deterioration, and most of them subsequently desquamate with denudation (Brody et al. 1972; Dewanjee et al. 1984). These changes in the venous graft are thought to be brought about by various factors: graft wall ischaemia (Brody et al. 1972), mechanical injury including overdistension (Ramos et al. 1976), loss of vasa vasora (Brody et al. 1972; McGeachie et al. 1981), and the shear stress (Friedman et al. 1987) or intramural stress (Thubrikar et al. 1988) of arterial circulation. Endothelial deterioration and repair of the grafts early after implantation may have a potent influence on the subsequent intimal thickening. Although some studies have reported on the fate of the endothelium in vein grafts (Brody et al. 1972; Dilley et al. 1983;

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Jones et al. 1973), the following questions are unresolved: what is the distribution of the surviving endothelial cells (as a source of re-endothelialization), and what is the relationship between endothelial regeneration and successive intimal lesions? The purpose of this study is to define what alterations occur in vein grafts-with and without storage in preparative solutions-early after implantation into the arterial circulation, and how these events influence the following intimal thickening in canine models. The results demonstrate that some endothelial cells in the vein graft are always preserved at the back of valves and that the majority of the regenerated endothelium is mainly derived from these survivors. Earlier endothelial repopulation reduces the subsequent intimal thickening.

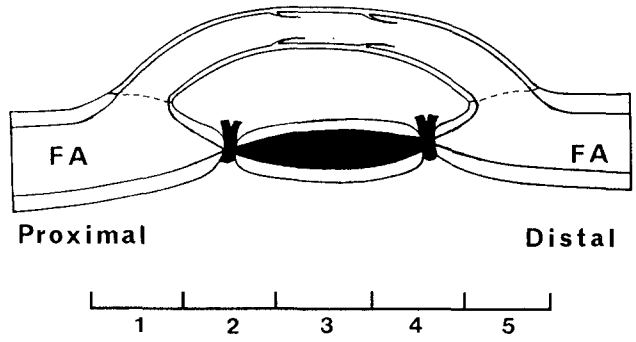

Fig. 1. Diagram of autologous saphenous vein graft in canine femoral artery (FA). Anastomosis is performed in an end-to-side fashion. Note that the graft is designed to have two valves in its middle. The numbers show a subdivision of the specimens distinguishing their location in the graft

Materials and methods

Thirty-nine male mongrel dogs, each weighing from 20 to 23 kg, were used. All animals received a non-lipid supplemented diet. The dogs were sedated by subcutaneous administration of ketamine chloride, 12 mg/kg, followed by intravenous injection of sodium pentobarbital, 25 mg/kg. An autologous vein graft, about 7 cm in length, was carefully harvested from the saphenous vein of a dog. Under a monitoring pressure of less than 100 mmHg measured with a transducer (Nihon Kohden, S-0411), all vein grafts were perfused with 0.9% sodium chloride solution containing 80 mg/dl of papaverine, which was used to prevent constriction of the grafts, and 4000 U/L of heparin (storage solution). In group 1 ($N=13$), no storage of the grafts was employed, while in group 2 ($N=26$) the grafts were kept in the storage solution for 1 h at room temperature. After injection of heparin 100 U/kg into the femoral artery, a reversed saphenous vein segment of about 5 cm length was implanted into a femoral artery in an end-to-side anastomosis. All grafts were designed to have two valves in their middle (Fig. 1). During the operation, the grafts were intermittently perfused with the storage solution to prevent drying. The duration that the grafts were exposed to ischaemia was about 40 min and 100 min in group 1 and group 2, respectively. The bypassed femoral artery was ligated with nylon ligatures at two anastomoses (Fig. 1). Using an overdose of pentobarbital, the animals were killed at 1 h ($N=3$ and 4), 1 day ($N=3$ and 4), 7 days ($N=3$ and 7), and 30 days ($N=4$ and 11) in group 1 and group 2 respectively after graft implantation. No occlusive thrombus formation was encountered in every experimental period and in both groups. All grafts were submitted to perfusion fixation with 3% glutaraldehyde-0.1 M cacodylate buffer solution (pH 7.4) at a pressure as low as 100 mmHg. The specimens were circumferentially divided into 5 segments, which were numbered from the proximal to the distal anastomosis (Fig. 1). The distal portion (approximately 2 mm in width) of each segment was examined immunohistochemically. Each segment remained was divided longitudinally into two parts, and one was submitted to transmission electron microscopy and the other was to scanning electron microscopy.

The immunohistochemical examination was made with the avidin-biotin peroxidase complex (ABC) method (Hsu et al. 1982) using rabbit mnonospecific anti-dog fibrinogen IgG (Ishii 1988). Sections were deparaffinized and incubated with rabbit anti-dog fibrinogen IgG (3 μg/ml) in phosphate buffered saline (PBS), pH 7.4. Endogenous peroxidase activity was blocked by

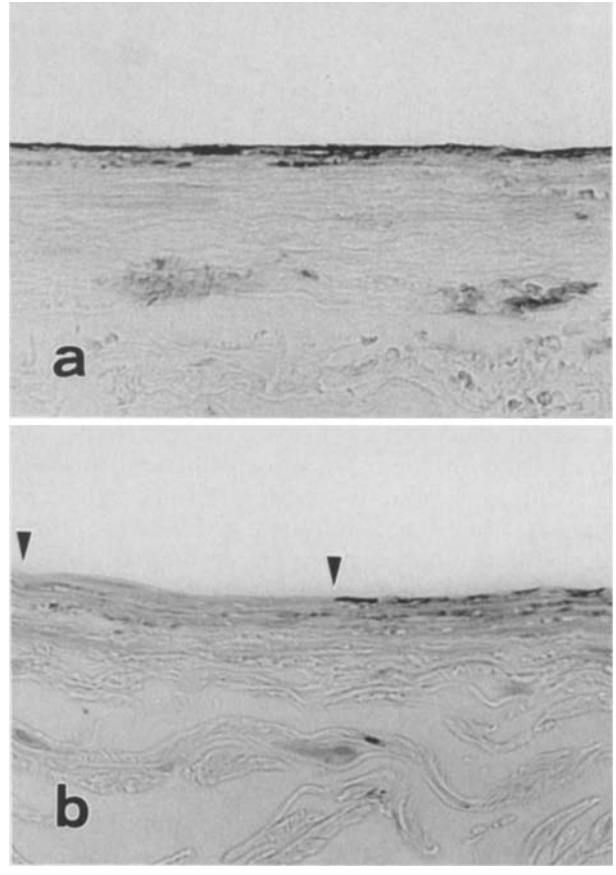

Fig. 2. a Immunohistochemical findings using rabbit anti-dog fibrinogen IgG 1 h after transplantation in group 2. Diffuse superficial and focal subendothelial reactions are noted. Endothelial cells are completely denuded. × 50. **b** Immunohistochemical finding using rabbit anti-dog fibrinogen IgG 1 week after transplantation in group 2. Positive reaction is diffusely observed on the graft surface and in the intima where endothelial cells are denuded, but faint reaction is found only in the subendothelium under regenerated endothelial cells (*between arrow heads*). × 200

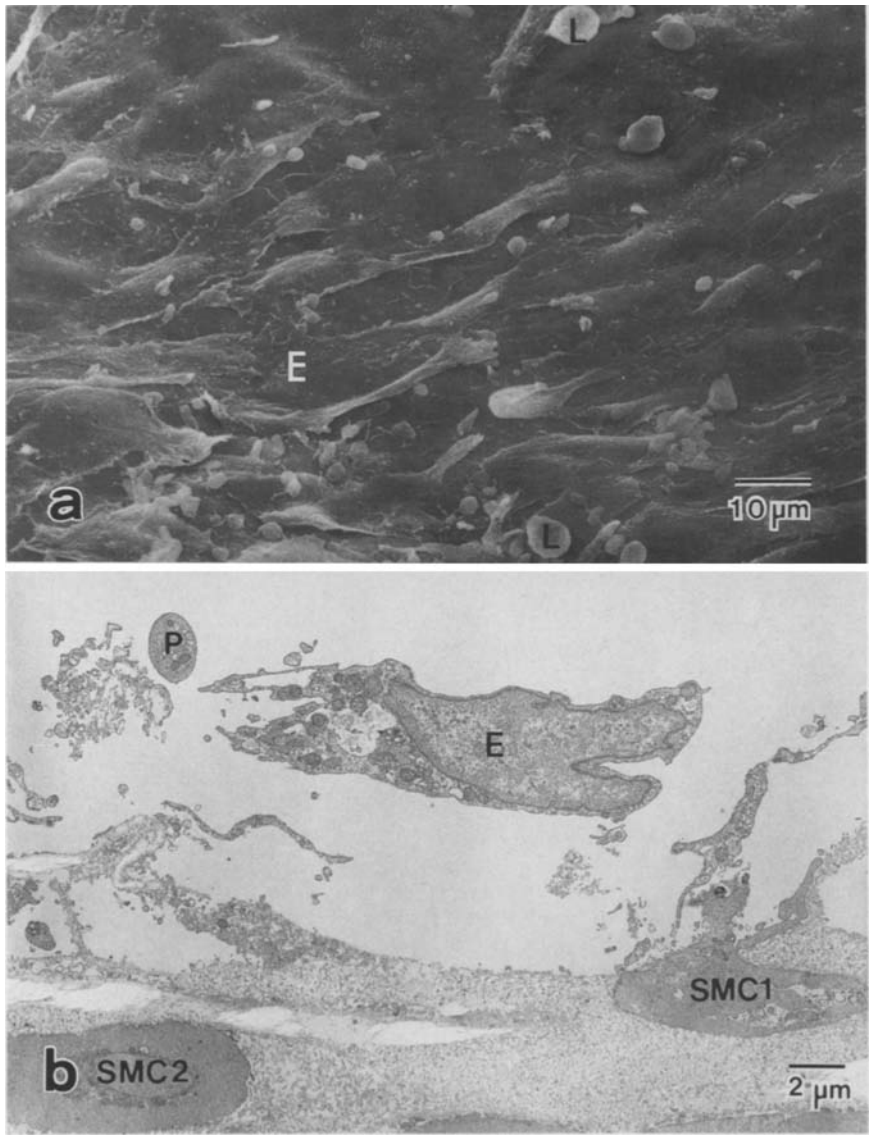

Fig. 3a. Scanning electron micrograph 1 h after transplantation in group 1. Graft surface is covered by degenerative endothelium (*E*) showing an increased electron reflection, shrinkage, gap formation and protrusion into the lumen with partial loss of their attachment. Platelets and leukocytes (*L*) adhere to the disrupted junctions and denuded subendothelium. × 600. **b** Transmission electron micrograph 1 h after transplantation in group 1. Endothelial cell (*E*) shows degenerative changes such as vacuolated endoplasmic reticulum and partial desquamation. A platelet (*P*) locates in the close vicinity of the disrupted junction. Smooth muscle cell (*SMC1*) of the innermost layer also exhibits degeneration, but the smooth muscle cell in the deeper wall (*SMC2*) appeared almost intact. × 3000

0.3% solution of H_2O_2 in methanol. The developing reagent used was 0.05%, 3,3′-diaminobenzidine-tetrahydrochloride (DAB) in PBS. Sections were subsequently counterstained in methylgreen.

For transmission electron microscopy specimens cut into pieces approximately 2 × 2 mm in size were postfixed with 2% osmium tetroxide for 2 h. After dehydration in alcohol series, they were embedded in Epon 812, half of them longitudinally and the others transversely. Semithin sections stained with toluidine blue were submitted to the examination of intimal thickness and incidence of thrombus formation. The ultrathin sections were cut with a diamond knife, followed by double staining with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX transmission electron microscope at 60 KV.

For scanning electron microscopy specimens were postfixed with osmium tetroxide for 2 h. After dehydration in alcohol series the specimens were immersed in isoamyl acetate and critical-point dried with carbon dioxide (HCP-2, HITACHI), followed by sputter-coating with platinum palladium. The specimens were examined with a JEOL JSM-34CF scanning electron microscope at 15 KV.

Results

At one hour and one day after implantation, immunostaining revealed a linear positive reaction for fibrinogen and/or fibrin on the intimal surface and focally in the subintima, where endothelial cells were denuded (Fig. 2a). Near the anastomotic points, the reaction was stronger and distributed in the deeper part of the intima. No significant difference in the staining pattern was revealed between one hour and one day after implantation. One week after implantation, the re-endothelialized intima showed an occasional faint reaction

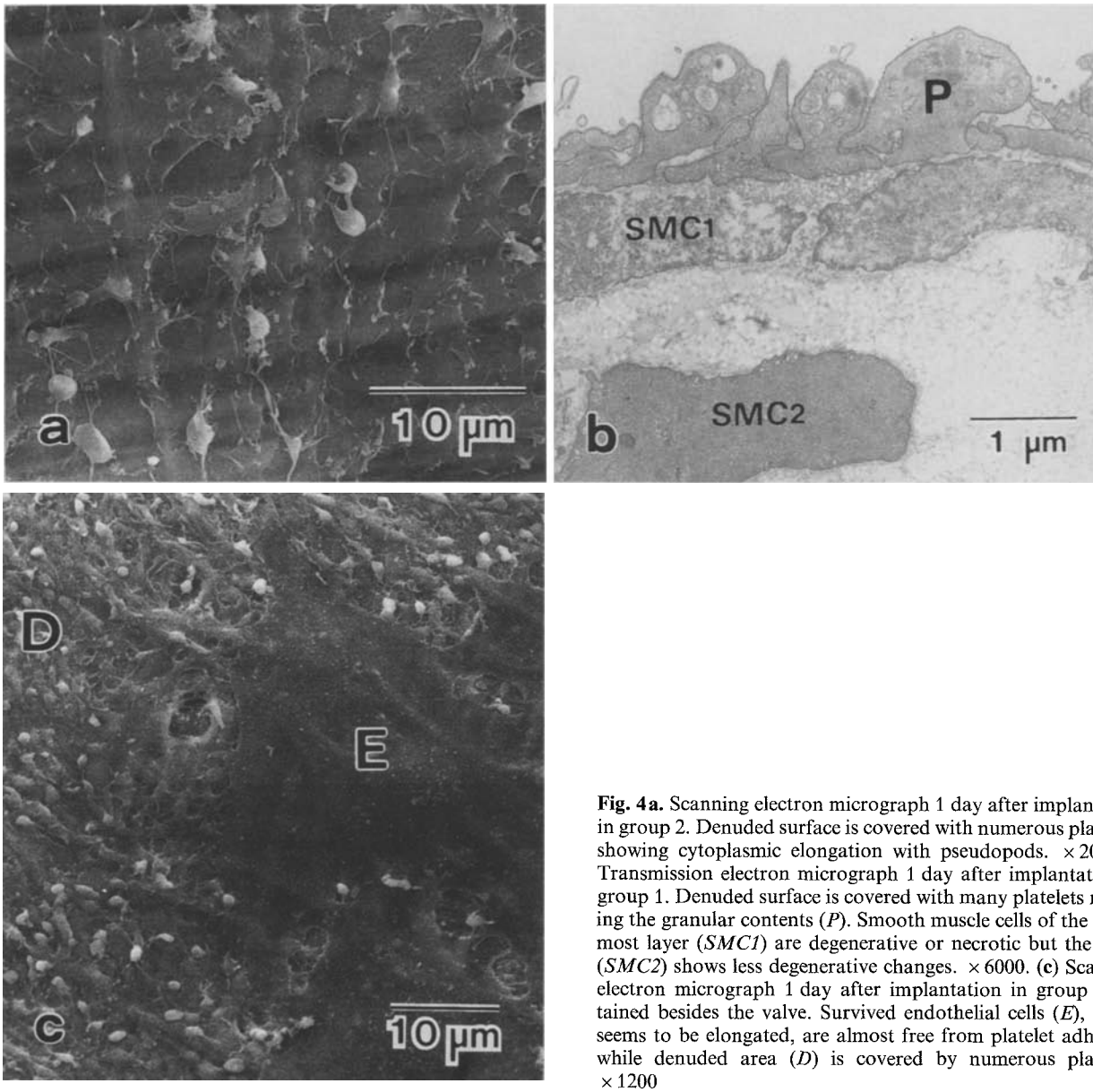

Fig. 4a. Scanning electron micrograph 1 day after implantation in group 2. Denuded surface is covered with numerous platelets, showing cytoplasmic elongation with pseudopods. ×2000. **b** Transmission electron micrograph 1 day after implantation in group 1. Denuded surface is covered with many platelets releasing the granular contents (*P*). Smooth muscle cells of the innermost layer (*SMC1*) are degenerative or necrotic but the other (*SMC2*) shows less degenerative changes. ×6000. (**c**) Scanning electron micrograph 1 day after implantation in group 1 obtained besides the valve. Survived endothelial cells (*E*), which seems to be elongated, are almost free from platelet adhesion, while denuded area (*D*) is covered by numerous platelets. ×1200

in the subendothelium, while there was a positive reaction both on the graft surface and in the subendothelium where endothelial cells were still absent (Fig. 2b). The sections which contained valvular tissue where the surface was largely lined by endothelial cells, always showed rare and weak positive staining both superficially and subendothelially. One month after implantation, a strong positive reaction was limited in the narrow regions where the endothelial cells were denuded, but a focal, faint reaction was observed in the intima despite an endothelial covering.

One hour after implantation, the majority of the endothelial cells exhibited morphological changes on scanning electron microscopy indicating endothelial damage or degeneration, such as an increase in electron reflection, shrinkage, and protrusion into the lumen with partial loss of attachment to the subendothelium (Fig. 3a). The denuded areas of endothelial cells revealed adhesion of platelets and leukocytes (Fig. 3a). The arterial endothelium closely adjacent to the vein graft was denuded and the subendothelium was also covered by platelets. One day after implantation, the degenerative endothelial cells seen at 1 hour had almost completely disappeared from the graft surface, and these areas were covered with platelets (Fig. 4a). However, on the back of the valves, islands of the

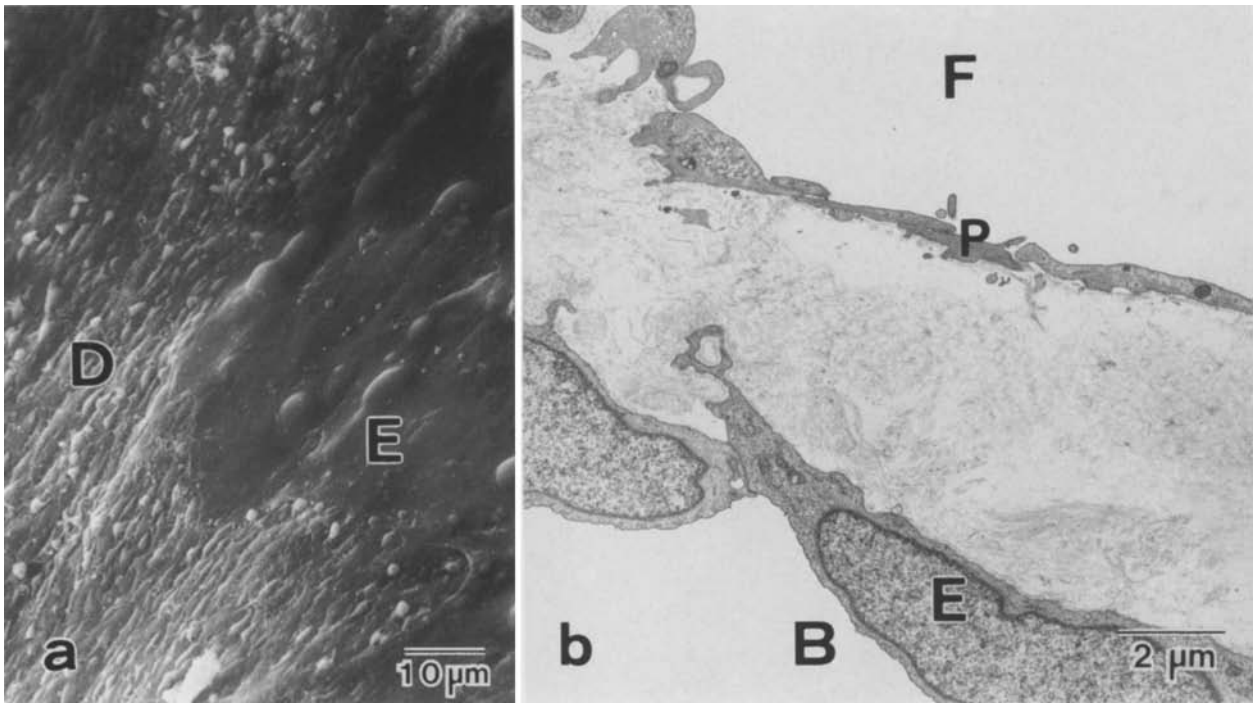

Fig. 5a. Scanning electron micrograph 1 day after implantation in group 1 obtained from the back of a valve. The back of a valve covered with endothelium (*E*) is completely free from platelet adhesion, while denuded area (*D*) is covered with platelets. ×680. **b** Electron micrograph 1 day after implantation in group 1. The back (*B*) of a valve shows remaining of endothelium (*E*), which seems to be morphologically intact, while the front (*F*) shows endothelial denudation and platelet (*P*) adhesion. ×4000

endothelial cells were always preserved, in both groups and were not associated with the adhesion of platelets or leukocytes (Fig. 5a). In group 1, besides these endothelial cells sticking on the valves, tiny islands of endothelial cells were scattered on the graft surface away from the valves, mainly in segment 2 and 4, but they exhibited an altered morphology, namely an increase in the number of microvilli, marginal foldings and gap formation between endothelial cells, and flattening (Fig. 4c). The area ratio of these islands to the entire luminal surface was $4.6 \pm 4.0\%$ ($N=3$, mean $\pm$ SD). No surviving endothelium was encountered on the graft surface of segment 1 and 5 which contained anastomosis. In group 2, however, the endothelium was denuded on almost the entire surface except for the back of the valves. Although these surviving endothelial cells showed various degrees of cell damage, no adhesion of platelets or leukocytes was apparent.

One week after implantation, new endothelial covering had progressed considerably on the graft surface, especially around the valves (Fig. 6a). The area of re-endothelialization of all segments was examined from the proximal to the distal anastomosis and the area ratio of the re-endothelialization to the whole area of the luminal surface was estimated. It resulted to be $71 \pm 14.7\%$ (mean $\pm$ SD, $N=3$) and $47 \pm 9\%$ (mean $\pm$ SD, $N=3$) in group 1 and group 2, respectively. Most regenerated endothelial cells were orineted parallel to the blood flow, but endothelial cells at the proliferating edges showed an irregular arrangement. A few platelets and leukocytes adhered to these endothelial cells and leukocytes infiltrated into the gaps between them. The arterial surface near the anastomosis was fairly well covered by endothelial cells, while the graft surface just adjacent to the anastomosis was scarcely covered by endothelium (Fig. 6b).

One month after implantation, the graft surface including the anastomosis was almost completely covered by endothelial cells, and they were confluent with each other and appeared morphologically to be almost normal (Fig. 8a).

One hour after implantation, most endothelial cells showed morphological alterations on transmission electron microscopy such as vacuolated endoplasmic reticulum, pyknotic nucleus, increased electron density of cytoplasm, disrupted cytoplasmic membrane and detachment from the subendothelium (Fig. 3b). One day after implanta-

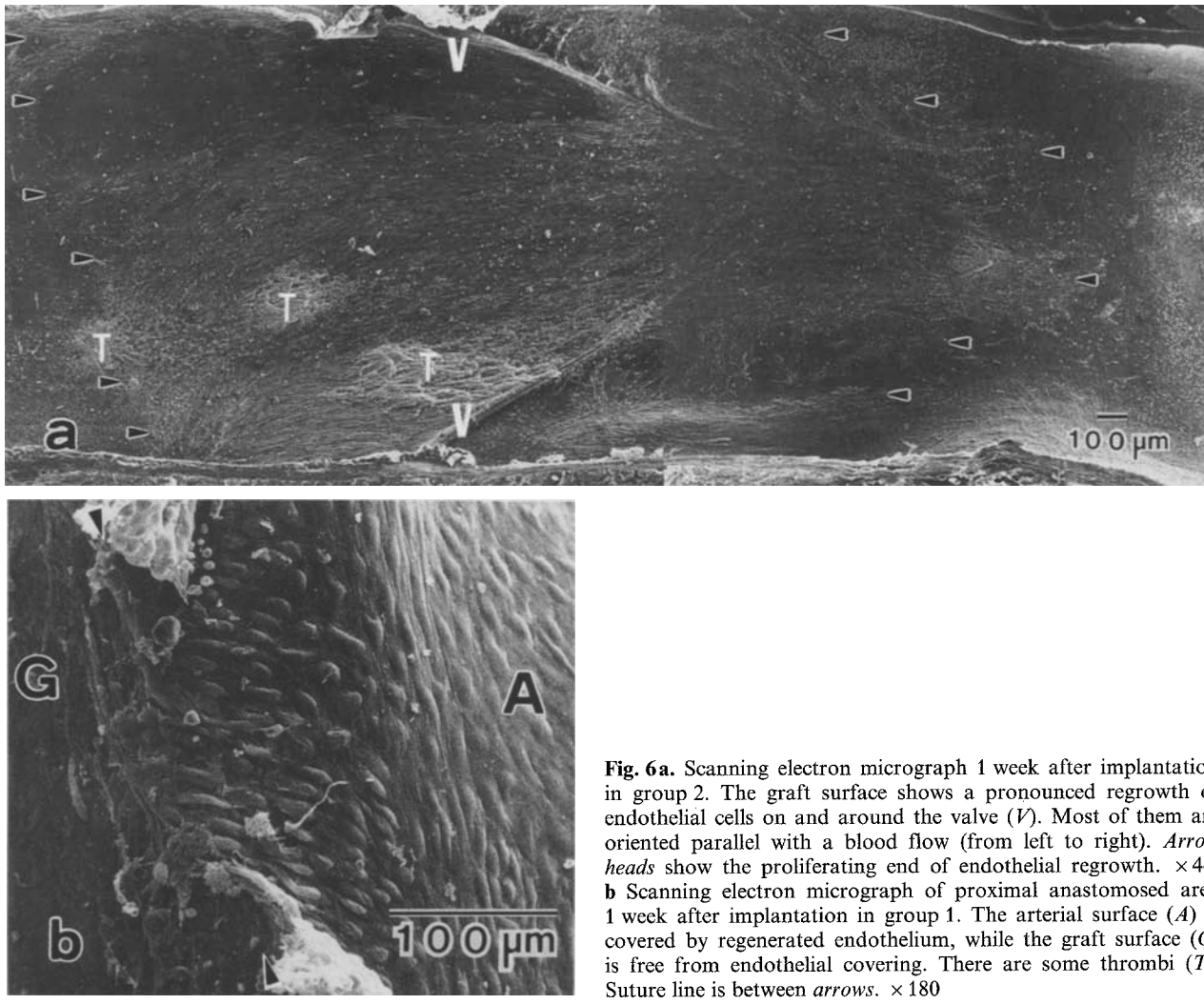

Fig. 6a. Scanning electron micrograph 1 week after implantation in group 2. The graft surface shows a pronounced regrowth of endothelial cells on and around the valve (*V*). Most of them are oriented parallel with a blood flow (from left to right). *Arrow heads* show the proliferating end of endothelial regrowth. ×44. **b** Scanning electron micrograph of proximal anastomosed area 1 week after implantation in group 1. The arterial surface (*A*) is covered by regenerated endothelium, while the graft surface (*G*) is free from endothelial covering. There are some thrombi (*T*). Suture line is between *arrows*. ×180

tion, 86% of the sections examined (52/60) showed complete endothelial denudation. The denuded graft surface was covered with platelets releasing the contents of granules (Fig. 4b). As observed by scanning electron microscopy, endothelial preservation was encountered on the back of the valves in both groups (Fig. 5b). Several sections besides the valves in group 1 show small remnants of endothelial cells, which showed a variety of cell damage such as increased electron density, cytoplasmic vacuolation and mitochondrial swelling. Red blood cells, leukocytes, and amorphous material were observed in the intima and partly in the media. Smooth muscle cells in the intima and the inner media showed degenerative or necrotic changes, but these changes seemed to be less marked in the deeper media (Fig. 4b).

One week after implantation most of the covering endothelial cells showed some characteristics of a regenerating nature such as non-interdigitating junctions, cytoplasm rich in organelles including mitochondria and rough endoplasmic reticulum and tightly packed filaments (Fig. 7). Denuded areas were still covered with platelets. Tiny mural thrombi were occasionally observed and they were composed of considerable amount of fibrin strands. Calculating the incidence of the thrombus formation examined in semithin sections, more than about 50 μm in size, in the respective segment, the incidence was most frequent (57%, 25/44) in the anastomosing area (segment 1 and 5) and least frequent (33%, 12/36) in segment 3 of both groups. Comparison between the two groups resulted to be more frequent in group 2 (58%, 47/85) than in group 1 (34%, 23/72) of the entire graft. Thrombus formation was also found in the valve pocket in six of the twelve sections of valvular tissue. Fibrin strands were also noted in the intima inter-

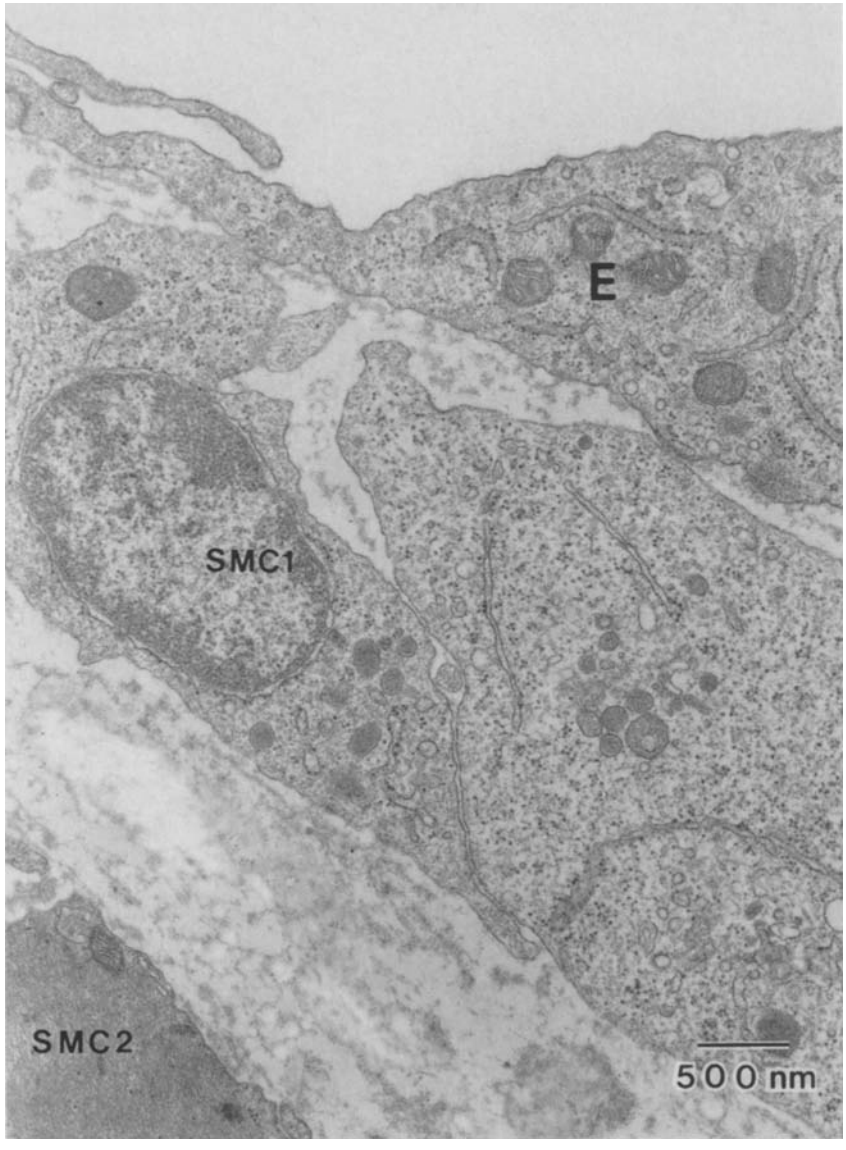

Fig. 7. Transmission electron micrograph 1 week after implantation in group 1. Regenerated endothelium (*E*) covers the graft surface. Smooth muscle cells (*SMC1*) in the inner most layer are in a synthetic state, while a smooth muscle cell (*SMC2*) in the deep layer is in a contractile state. Note the rich organelles in the cytoplasm of regenerated endothelium. ×10000

mingled with a few platelets and other blood components. The majority of the smooth muscle cells in the intima and the inner media showed a synthetic type morphology (Fig. 7). Several synthetic smooth muscle cells migrated and proliferated in these thrombi.

One month after implantation, the covering endothelial cells had become rich in intracytoplasmic organelles and the stress fibers had increased in number (Fi. 8b). The intima showed various degrees of intimal thickening composed of smooth muscle cells in either the contractile or the intermediate type (Fig. 8b) and abundant extracellular matrices including collagen fibers, proteoglycans, and elastic fibers. Some areas of the grafts showed an uneven surface, namely a protruding area with abundant collagen fibers and scanty intimal smooth muscle cells, and a hollow area with a considerable number of intimal smooth muscle cell layers (Fig. 9a, b).

Intimal thickness was examined by counting the number of intimal cell layers in the semithin sections (400 sections) obtained from each segment in light microscopy. One week after implantation the intima showed a thickening of at most three cell layers oriented parallel with the axis of blood flow, but no significant difference was observed between each separate segment. One month after implantation the intimal thickening was apparently less in the middle of the grafts than in other areas, while pronounced thickening was observed in the vicinity of the anastomoses in both groups

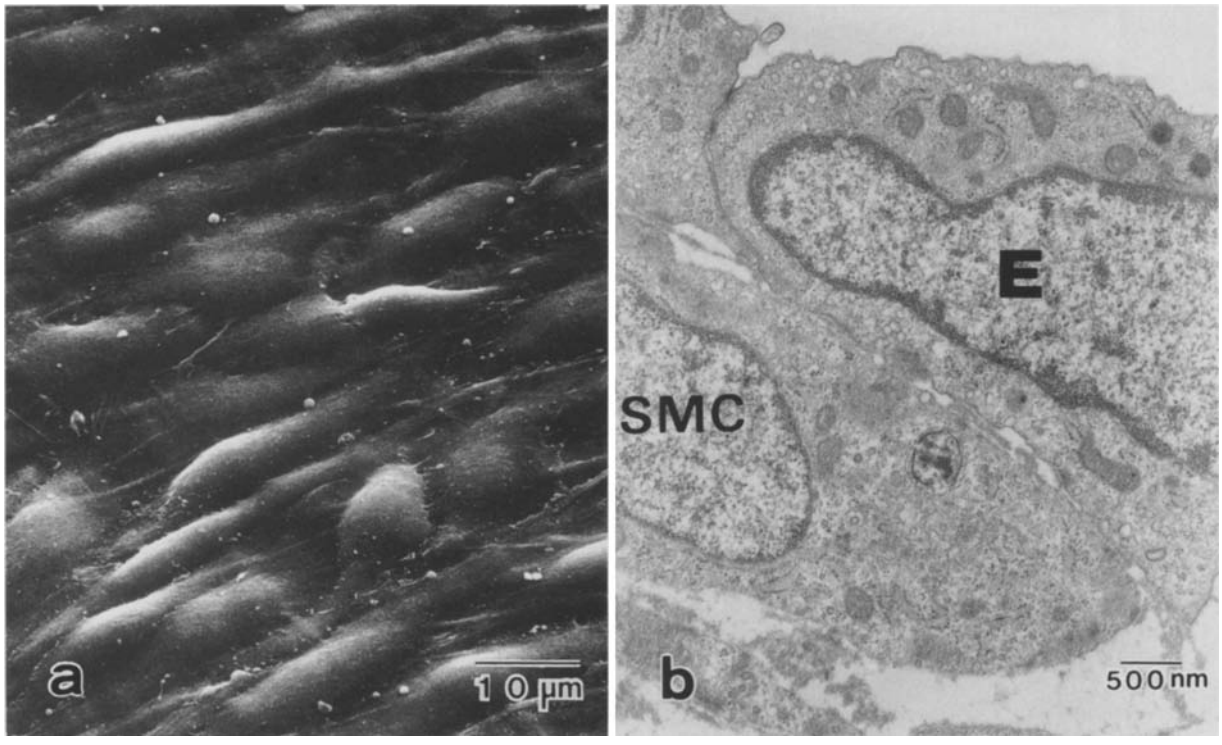

Fig. 8a. Scanning electron micrograph 1 month after implantation in group 2. Confluent monolayer of regenerated endothelium covers the graft surface. ×1100. **b** Transmission electron micrograph 1 month after implantation in group 1. Endothelial cells (*E*) is rich in mitochondria, rough endoplasmic reticulum, and microfilaments. Smooth muscle cell (*SMC*) increases its microfilaments, which indicates the intermediate or contractile state. ×10000

(Fig. 10). Comparing the two groups, the intimal thickening at the segments of No. 2 and No. 3 in group 2 was more severe than that of the respective segments in group 1 (Fig. 10).

Discussion

The data presented here show that the re-endothelialization of the graft surface started mainly from the valves located in the middle of the graft and spread rapidly upstream, downstream and circumferentially, ultimately conjoining with the endothelium which regenerated slowly from the adjacent artery after one month. This indicates that the endothelial cells remaining on the back of valves at day one were a major source of re-endothelialization of the vein grafts. The reason why these cells were saved from denudation may be that the back of the valve is less affected by arterial blood flow. The time spent before transplantation influences the severity of endothelial damage induced both before and after transplantation, and also affects the rate of the following endothelial repopultion. In fact, in group 1, without storage, the endothelial cells remained in areas away from the valves, while such cells were not aparently encountered in group 2 even with 1-h storage. The persistent endothelial cells in group 1 might be an other additional source of re-endothelialization. Some investigators have reported that the endothelial cells of the adjacent artery also migrated to the graft surface early after implantation of both autologous vein grafts (Dilley et al. 1983) and prosthetic grafts (Clowes et al. 1985; Lei et al. 1987). In contrast to other findings, our data demonstrated that the arterial endothelium did not progress across the anastomosis by one week. The differences in anastomosing style and animal species (Reidy et al. 1986) might cause the difference in results. A possible explanation for the delayed re-endothelialization at the anastomosis is that the deep injury caused by surgical manipulation at the anastomosis inhibits an endothelial regrowth. Walker and his colleagues (1983) have reported that a deep arterial injury made with nylon catheter resulted in a slower regrowth of the endothelium than a superficial injury. A second possibility is that turbulent flow at anastomosing areas (Thubrikar et al. 1988) might affect the repopulation of the grafts by the arterial endothelium.

The proliferation of smooth muscle cells in the intima became apparent at 1 week. They were the

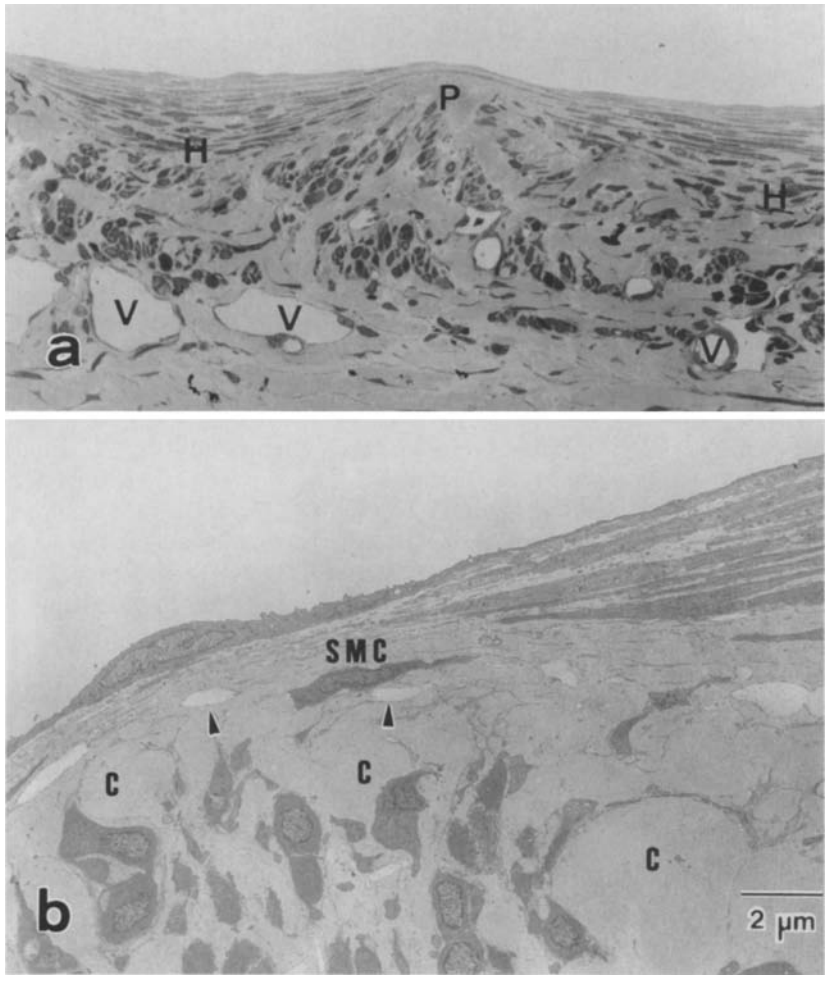

Fig. 9a. Longitudinal section obtained from a segment of No. 3 1 month after implantation in group 2. Note that there is a protruding area (*P*) alternatively with adjacent two hollowing areas (*H*). The hollows show a considerable intimal thickening, while there is no intimal thickening at the top of the protruding area. Some vasa vasora (*V*) are also indicated. (Toluidine blue stain, × 50). **b** Transmission electron micrograph of the same region as is shown in Fig. 9a. The protruded lesion is composed of large immature collagen bundles (*C*) and medial smooth muscle cells. A smooth muscle cell (*SMC*) is migrating into the intima through a fenestra of elastic lamina (*arrow heads*). × 1200

synthetic type morphology, suggesting that their division could be stimulated by mitogens and that they were synthesizing secretory protein (Campbell and Campbell 1981; Campbell et al. 1981). Ross and Glomset (Ross and Glomset 1973) reported that phenotypic modulation of medial smooth muscle cells was supposed to be a prerequisite for their subsequent proliferation in the intima, after migration through fenestrae of internal elastic lamina. However, elastic fibers in the vein are thin and there are wider fenestrae between inner elastic laminae. Activated medial smooth muscle cells in veins, therefore, seem to migrate more easily into the intima than those in the artery.

The intimal thickening observed at one month was less in the middle of the grafts than in the other locations. This difference was well correlated with the time course of re-endothelialization. These findings indicate that endothelial regeneration is important as an inhibitory regulating factor for smooth muscle cell proliferation, probably by protecting the vascular wall from the effects of growth factors like PDGF (Ross 1981; Ross and Vogel 1978) and by secreting a heparin-like material (Fuster and Chesebro 1986). In contrast, the anastomotic sites of the vein grafts showed the most prominent intimal thickening and the slowest regrowth of endothelial cells, possibly indicating that a prolonged endothelial loss was a major causative factor inducing the noticeable proliferation of smooth muscle cells in the intima. Similar findings have been reported by several investigators (Jones et al. 1973; Lawrie et al. 1976) and the recurrent endothelial injury was partly attributed to arterial blood flow (Jones et al. 1973). The effect of wall shear stress (Friedman et al. 1987) may be implicated to the prominent intimal thickening of the anastomoses. The influence of the shear stress is also strongly suggested by our finding of the uneven intimal thickening away from anastomosis at one month (Fig. 9a, b).

Our results support the findings of Hauden-

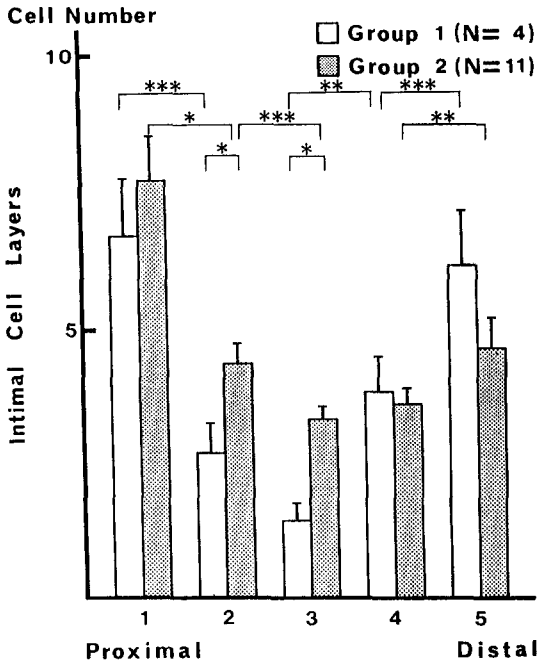

Fig. 10. Comparison of intimal thickening between group 1 and 2 in each segment at 1 month. The number of the semithin sections examined was 21 and 33, 28 and 77, 30 and 62, 29 and 64, and 22 and 34, in group 1 and group 2, and from segment 1 to segment 5, respecitvely. *Bars* represent standard errors. The Wilcoxon test for unpaired observation was used. * $p<0.005$, ** $p<0.05$, *** $p<0.025$

schild and Schwartz (1979) that after balloon injury to rat aorta regions rapidly repopulated with endothelium showed less intimal thickening than those slowly repopulated. Recently, however, Tada and Reidy (1987) have suggested that the migration of smooth muscle cells into the intima might be controlled by factors other than PDGF. Gajdusek and Schwartz (1984) have reported that lysates of smooth muscle cells, dermal fibroblasts, and endothelial cells contain a cytoplasmic mitogen distinct from PDGF. The degenerative and the necrotic smooth muscle cells which were observed in the inner wall of the grafts by one week may provide additional smooth muscle mitogenic activity in our experiments.

It is still unclear why smooth muscle cells of the inner wall suffered more severe deterioration when compared with those of the deeper wall. It may be possible to explain the vulnerability of the inner smooth muscle cells by considering the following factors: (1) reperfusion injury, (2) cytotoxic substances permeating from blood components which may injure the smooth muscle cells, and (3) excessive extension of the graft wall by arterial pressure.

Our immunohistochemical findings revealed a less deposition of fibrinogen and/or fibrin on the graft surface and in the subendothelium with an endothelial covering than in the denuded area, and electron microscopic findings showed a less frequent thrombus formation in the middle of the grafts than in the anastomotic sites of the grafts. Furthermore scanning electron microscopic findings elicited a wide endothelial covering around the valves locating in the middle of the grafts at one week after implantation. These findings suggest that deposition and permeation of fibrinogen and/or fibrin might stimulate the proliferation of vascular smooth muscle cells (Ishida and Tanaka 1982) and might play an atherogenic role in the intima of the vein grafts (Brody et al. 1972; Jones et al. 1973) as well as of the artery (Sadoshima and Tanaka 1979). Thus it can be said that an earlier endothelial covering, especially in the segment No. 3, resulted a less intimal thickening partly because of a less deposition of fibrin and/or fibrinogen in our experiments. The thrombus formation boserved in the valvular region at 1 week might be referable to a valve related stenosis at late time (Fuchs et al. 1978), but our observation of short period can not elicit any explanation about what is the major contributor to the valve related stenosis.

We conclude that the venous valves play an important role in re-endothelialization of the graft surface and that the rate of the endothelial regrowth correlates inversely with the degree of subsequent intimal smooth muscle cell proliferation. It is possible to say that the degree of the graft damage before and early after implantation is one of the most important factors which decide the ultimate fate of the grafts.

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