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Recovery of Endothelial Cells and Prostanoid Production in Endothelial Cell-Seeded Grafts

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Objective: *To investigate the function and morphology of endothelial cell (EC) seeded grafts.*
Design: *Experimental, open study.*
Chief outcome measures: *Endoluminal release of prostacyclin (6-Keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2), patency, EC coverage and cell identity.*
Materials: *In 12 sheep, segments of both carotid arteries were excised. On one side a seeded and on the other an unseeded dacron graft were inserted. After 3 months the grafts were excised. In grafts and arteries, the endoluminal release of 6-keto-$PGF_{1\alpha}$ and TxB_2 was determined in a perfusion system. Scanning electron microscopy (SEM) and light microscopy were used to determine the EC coverage and cell identity.*
Results: *Eight animals survived. Three seeded and two unseeded grafts were occluded. Prostacyclin release did not differ significantly between seeded and unseeded grafts and arteries, when the arteries were looked upon as one group. When the graft was compared with its corresponding artery, i.e. the artery it replaced, a significantly lower release was found in the unseeded group. Thromboxane release was undetectable in arteries but significantly higher in both graft groups. SEM revealed a cellular coverage of 75% in the seeded grafts and 50% in the unseeded (not significant). Light microscopy showed a patchy staining for Factor VIII-related antigen in some grafts in both groups.*
Conclusion: *Prostacyclin release in unseeded and seeded dacron grafts did not differ 3 months after implantation in sheep, except when the graft was compared with its corresponding artery. The significance of this remains to be settled. Seeded grafts did not have a higher proportion of endothelial coverage than unseeded grafts.*

Key Words: Blood vessel prosthesis; Endothelium; Prostacyclin (PGI_2); Sheep; Thromboxane (TxA_2).

Introduction

The ability of prosthetic vascular grafts to maintain patency is inferior to that of the native artery and vein grafts. This may depend on the fact that the prosthetic vascular graft does not heal with a true endothelial cell lining. The concept of endothelial cell-seeding was introduced in an attempt to create an intimal lining which has properties similar to that of the native artery. Canine experiments have shown that seeded endothelial cells produce an intimal lining in the graft and that such grafts have a better patency than unseeded grafts.[1] One difficulty in evaluating this process lies in the fact that there are species differences in the healing of synthetic grafts. Another difficulty is the lack of functional "tests" of the healed graft.

The endothelial cell is involved in the healing process of a traumatised artery.[2] It also produces a large number of active substances which act or interact with others to keep the vessel wall free from blood clot.[3,4] One of these substances is prostacyclin (PGI_2) which is a potent inhibitor of platelet aggregation. The new intima in grafts, either seeded or unseeded, produces PGI_2 in small amounts. These measurements have usually been made from punch biopsies[5] or after luminal incubation.[6] Another technique is to use an artificial circulation with physiological flows and pressures to give a better approximation of the *in vivo* situation. This also increases PGI_2 release because of the presence of shear stress. In addition, the perfusion system only communicates with the luminal area and not with the deeper layers of the graft tube.[7–10] Production of PGI_2 could possibly contribute to decreased thrombogenicity, whereas a high thromboxane (TxA_2) release could have the opposite effect. In a previous experiment we have showed that, after 3 weeks, seeded grafts in sheep had a significantly higher release of PGI_2 than unseeded,

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although they did not reach the levels of the native artery.[11] The present experiment was designed to investigate the capacity of the seeded and unseeded grafts to produce prostacyclin after 3 months of implantation and to compare this with that of the native artery. As there was a significant difference after 3 weeks of implantation the 3 month time span was used to evaluate whether the differences at 3 weeks were further enhanced or not.

Material and Methods

Twelve male sheep weighing 25–34 kg were used and cared for according to the European Convention on Animal Care. The experiment was approved by the Animal Ethics Committee of Lund University.

Surgical procedure

The animals were anaesthetised with approximately 200 mg of thipental-sodium (Pentotal, Abbott Laboratories in North Chicago, Illinois, U.S.A.), intubated and ventilated with an oxygen/N_2O mixture. Anaesthesia was maintained with a continuous infusion of ketamin hydrochloride (Ketalar, Park-Davis, Morris Plains, New Jersey, U.S.A.) approximately 240 mg per hour combined with 10–20 mg diazepam (Apozepam, A/S Apothekernes Laboratorier, Oslo, Norway). During the operation the animal received 500 ml Ringer's solution. Antibiotics (Combiotic Vet containing dihydrostreptomycin: 250 mg/ml, benzylpenicillin: 200 mg/ml, and procaine hydrochloride: 20 mg/ml; Pfizer, New York, U.S.A.) were given in a dose of 3 ml once a day for 4 days, the first dose being given preoperatively.

A midline neck incision was made and the left jugular vein excised for endothelial cell harvest (see below). Both carotid arteries were dissected free and the tributaries ligated. Before clamping, 100 IU of heparin (Kabi Pharmacia AB, Stockholm, Sweden) per kg body weight was given. About 5 cm of the artery was excised. A 6 cm long and 6 mm diameter externally reinforced knitted double velour dacron graft (Microvel supported 100, Meadox Medicals, Oakland, New Jersey, U.S.A.) was inserted on each side using oblique anastomoses. On one side a preclotted and on the other a preclotted and endothelial cell-seeded graft were inserted. The side was chosen at random just before insertion. After 3 months the animals were anaesthetised and the grafts were dissected free, patency assessed, excised and then perfused as described below.

Preparation of endothelial cells

After careful dissection with ligation of all tributaries, 8–12 cm of the left jugular vein was excised and small cannulae (Gambro Lundia AB, Lund, Sweden) were introduced and tied at each end. The vein was flushed with phosphate-buffered saline (PBS, Nordvac Medica) and then filled with a solution of Dispase (Dispase II, neutral protease, Boehringer Mannheim, Mannheim, Germany, catalogue no. 165859) 5 mg/ml in PBS and incubated at 37°C for 60 min. The vein was emptied of the enzyme solution, filled with PBS and slightly massaged between fingers to loosen any residual cells. Both cell suspensions were centrifuged at 200 *g* for 7 min, the pellets were then washed in PBS in the same manner, suspended in 1 ml M-199 (Medium 199, Gibco Laboratories, Grand Island, New York, U.S.A.) and an aliquot counted in a Bürker chamber and viability assessed with trypan blue. One of the two preclotted grafts was occluded at one end with a clamp, filled with the cell suspension, topped up with M-199, and occluded at the other end. It was left for 45 min at 37°C to allow the cells to adhere and during this time it was rotated by 90° every 10 min. In order not to diminish the number of cells seeded, no subculturing was made. Cells harvested in an identical manner have been cultured and identified as endothelial cells by their expression of Factor VIII-related antigen.

Evaluation of prostacyclin and thromboxane release

The excised carotid arteries, as well as the grafts, were perfused with Hank's balanced salt solution (Gibco Laboratories, pH 7.4, 290 mOsm) in an *ex vivo* perfusion system at a pressure of 100 mm Hg, and a pulsatile flow of 100 ml/min for five 15 min periods. For the last period arachidonic acid (AA) was added (4 μg/ml). This method has previously been described in detail.[12,13] The perfusate from each period was frozen at –20°C and later assessed for prostacyclin and thromboxane content. Prostacyclin was determined as the stable degradation product 6-keto-$PGF_{1\alpha}$ using a radio-immunoassay kit (Amersham International, Amersham, U.K.) and expressed as pg/mm^2 of luminal surface/15 min. The cross-reactivity for other prostaglandins with this kit is less than 1%. Thromboxane A_2 was determined as its stable degradation

product thromboxane B_2 using radioimmunoassay (Amersham International, Amersham, U.K.). Release is expressed as pg/mm^2 of luminal surface/15 min. All samples were run in duplicates in one assay.

Histological examination

After perfusion the arteries and the graft specimens were perfusion-fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 100 mm Hg to minimise distortion. The graft was divided in four parts and alternately allocated to light or scanning electron-microscopic examination (SEM).

Grafts were studied with haematoxylin-eosin staining, and a polyclonal rabbit anti-human antibody at a dilution of 1/100 (Dakopatts, Copenhagen, Denmark, code No. A 082) was used to stain for FVIII-related antigen. The antibody was then visualised through an alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (Dakopatts No. D651). The antibody reacts with sheep Factor VIII-related antigen as shown in the sheep artery. For SEM, after perfusion fixation, the specimens were dehydrated in ethanol, incubated for 10 min in hexamethyldisilazane (HMDS), dried in air and sputter-coated with gold-palladium.[14] The specimens were evaluated without knowing whether the graft was seeded or not. From each graft five different areas were investigated. Endothelial cell tufts and cobble stone morphology were used as identification criteria for endothelium, and cell coverage was assessed as a percentage of the area seen at 150 × magnification.

Statistics

Results of prostanoid and thromboxane analysis are expressed as pg/mm^2 of graft luminal area/15 min and are presented as medians and quartiles. For comparison, the Wilcoxon signed rank test was used.

Results

A median of 200 × 10^3 cells (range 100–800 × 10^3) were harvested. The viability exceeded 95%. Four animals did not survive the 3 month period. Two animals died after the operation — one because of severe diarrhoea and the other because of aspiration. The other two died after 21 and 37 days, respectively, both because of graft infection on one side. Among the other eight animals there were three occlusions of seeded grafts and two occlusions of unseeded grafts. One animal had occlusion on both sides, the other occlusions were unilateral.

Prostacyclin and thromboxane release

When both graft groups were compared with the artery group, prostacyclin release did not differ significantly between seeded and unseeded grafts, and there was no difference between any of the graft groups and the arterial group (Fig. 1). When compared to their corresponding arteries, i.e. the artery they replaced, prostacyclin release was similar in the seeded group (Fig. 2). The unseeded grafts had similar release during the first three perfusion periods, but significantly lower during the last two periods ($p = 0.02$; fig. 3).

The release of thromboxane from native artery was extremely low, and it did not respond to addition of arachidonic acid, which was seen in the two graft groups (Fig. 4). At 75 min, both grafts differed significantly ($p = 0.005$) from native artery but not from each other.

Histology

The pseudointima varied in thickness, but was mostly

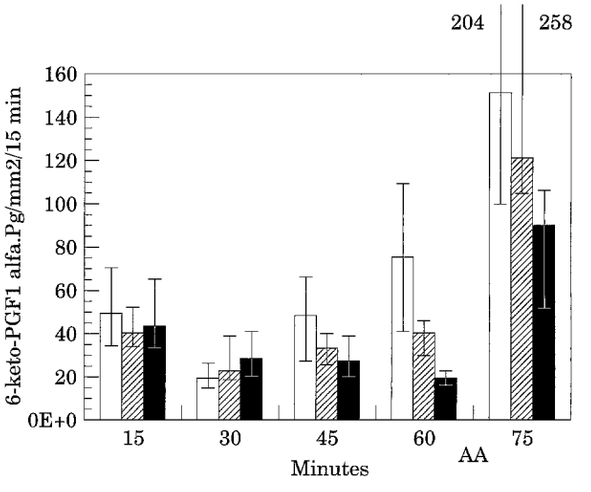

Fig. 1. Release of 6-keto-PGF$_{1\alpha}$ from native artery, seeded graft and unseeded graft, perfused *ex vivo* for five 15 min periods. AA = addition of arachidonic acid. Median and quartiles. (□) artery; (▨) seeded graft; (■) unseeded graft.

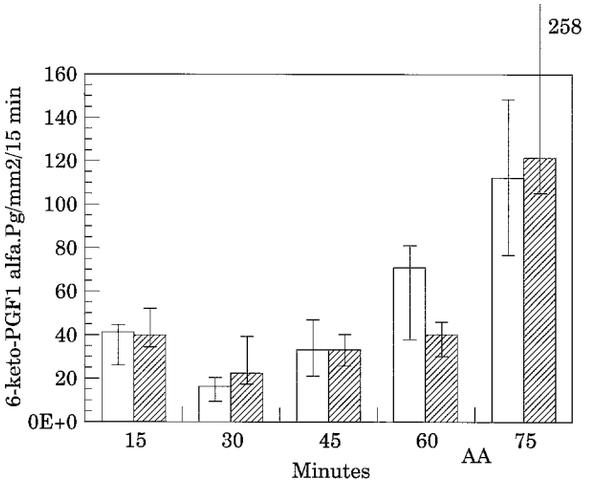

Fig. 2. Release of 6-keto-$PGF_{1\alpha}$ from seeded graft and its corresponding artery, i.e. the artery it replaced. Perfused *ex vivo* for five 15 min. periods. AA = addition of arachidonic acid. Median and quartiles. (□) corresponding artery; (▨) seeded graft.

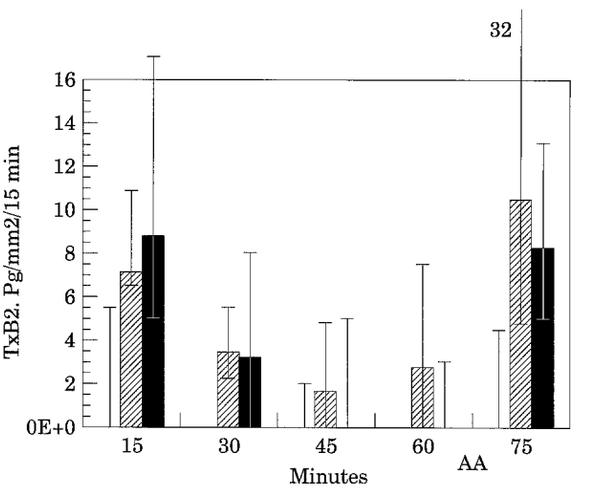

Fig. 4. Release of TxB_2 from native artery, seeded graft and unseeded graft perfused *ex vivo* for five 15 min. periods. AA = addition of arachidonic acid. Median and quartiles. (□) artery; (▨) seeded graft; (■) unseeded graft.

thin. Two of the seeded grafts, and three of the unseeded grafts showed a positive staining for Factor VIII-related antigen, but only in patches, although there could be a seemingly continuous cellular layer on the luminal side of the wall. SEM revealed endothelial cells (Fig. 5) in all grafts, but not necessarily in all five evaluated areas of each graft. There were also cells with characteristics of smooth muscle cells or fibroblasts (Fig. 6). The cellular coverage was a median of 75% (range 40–80) among the seeded grafts and among the unseeded grafts 50% (30–63; NS).

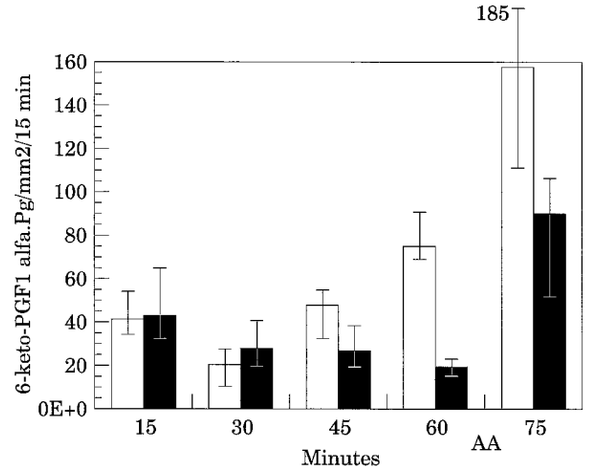

Fig. 3. Release of 6-keto-$PGF_{1\alpha}$ from unseeded graft and its corresponding artery, i.e. the artery it replaced. Perfusion *ex vivo* for five 15 min. periods. AA = addition of arachidonic acid. Median and quartiles. (□) corresponding artery; (■) unseeded graft.

Control native arteries showed a 90–100% coverage of the luminal surface by endothelial cells.

Discussion

Prostacyclin is a potent inhibitor of platelet aggregation. The production of PGI_2 might therefore be of benefit and show the functional quality of grafts in contrast to morphological methods. We have previously shown that the prostacyclin production in seeded grafts is significantly higher than in unseeded after 3 weeks of implantation.[11] The present experiment with grafts implanted for 3 months reveals that

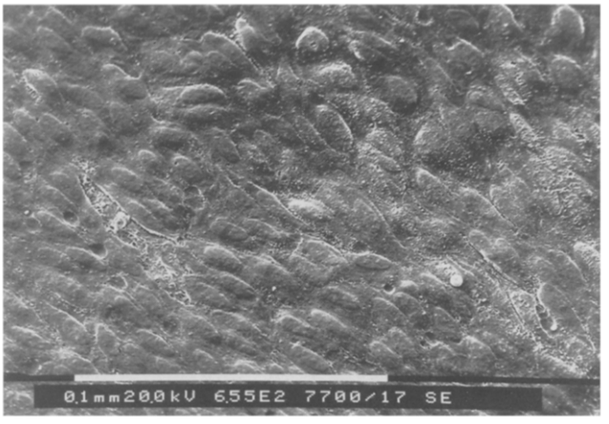

Fig. 5. SEM picture of endothelial cell-covered luminal surface. Original magnification × 655. Reproduced here at 50%.

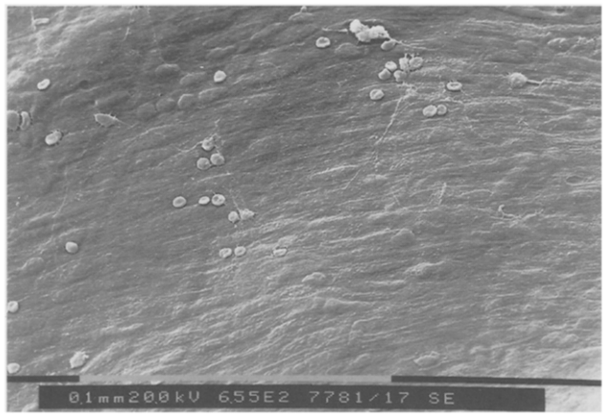

Fig. 6. SEM picture of smooth muscle cell-covered luminal surface. Original magnification × 655. Reproduced here at 50%.

the seeded grafts increased their ability to release prostacyclin to levels similar to those of the native artery. The unseeded grafts, which had almost no release at 3 weeks, also attained this ability after 3 months with no difference *vs.* seeded grafts, neither in basal release, nor in response to addition of arachidonic acid. Greisler *et al.*[15] found that the release of PGI_2 from an unseeded polyglactin graft in the rabbit reached aortic levels of release in 3 months. In this respect sheep and rabbits seem comparable. In rabbits subjected to balloon dilatation injury, the endothelium is destroyed. After 3 weeks the pseudointima is capable of releasing prostacyclin in amounts similar to that of the native artery.[16]

The comparison between the graft and its corresponding artery, i.e. the artery it replaced, is sensible since there is an inter-individual difference in PGI_2 release. However, the numbers were small. With this comparison there was a difference in the unseeded group during perfusion periods four and five. The interpretation of this is not obvious, but it certainly suggests that the functional properties of unseeded grafts are inferior compared with their corresponding artery.

Normally there is very low release of thromboxane from native arteries in the dog[8,13] and in the rabbit.[17] This was the case also in sheep. Thromboxane release is not easily stimulated in arteries,[12] but there was a significantly higher release after addition of arachidonic acid in both graft groups. This may depend on a mixed luminal cell population with different functional properties compared to native endothelium. Another possibility is that the diffusion through the healed graft wall was different from that of the artery. The assay has some cross-reactivity with arachidonic acid, but judged from the native artery group this is very low. There were no platelets left on the graft surface, which rules them out as the source of the measured thromboxane.

A healthy artery is not comparable to a prosthesis, but in the artery, attempts have been made to identify the cells or cell layers responsible for production of PGI_2 and thromboxane. Moncada *et al.*[18] found the highest rate of release of PGI_2 from the intimal surface layers and a decrease towards the adventitial region. The release from the free-dissected intima was around 37% of the total release from the arterial wall. Sinzinger *et al.*[19] studied punch biopsies and cell cultures and found, on the contrary, a higher percentage from the non-endothelial layers. Our own comparison of incubation *vs.* perfusion[7] shows that around 44% of the total vessel wall PGI_2 release comes from the luminal side. The corresponding figure for thromboxane is 16%.

The choice of species is important.[20] There is no ideal animal for thrombosis research. We choose the sheep on the basis of earlier experiments[11,21] and because their coagulation parameters are, in some ways, similar to those of humans.[22] In order to evaluate the endothelial cell seeding process we deliberately did not use any platelet aggregation inhibitor, although there is evidence that this might be beneficial in combination with seeding.[1] Other experiments have suggested that contrary.[23] There was no difference in patency, but the numbers were too small to draw any conclusions in this respect, and moreover this was not the primary aim of this experiment.

The morphological studies can, for practical reasons, only show a small sample of the total graft. Nevertheless they are helpful in the understanding of graft healing. From light microscopic criteria the endothelial cell coverage was patchy and scarce, and from SEM criteria it was more abundant. We could not find any correlation between the two methods. In contrast to our findings from the 3 week experiment,[11] at which time it was fairly easy to categorise the SEM findings due to a more or less all-or-nothing cellular covering, the 3 months of healing resulted in a high degree of cellular coverage, although the cell identification was not always easy. In humans vascular grafts heal with only limited ingrowth of endothelium,[24] so in this respect the sheep is a less accurate model of human characteristics.

PGI_2 release is only one of several regulatory factors where the endothelium is involved, but this analysis is an attempt at a more functional view of the healing graft. When summing up the results from 3 weeks of implantation[11] and 3 months it is fair to say that after 3 weeks the seeded grafts had a significantly higher PGI_2 release than the unseeded, but significantly

lower than that of the native artery. After 3 months no difference could be demonstrated between the native artery and the seeded graft, but the unseeded graft had a lower, possibly significant release. This suggests a functional difference, despite the long healing time. At 3 weeks, the seeded grafts had a better coverage, but at 3 months this had, in both types of grafts, evolved to a patchy coverage of endothelium and a population of fibroblasts. It thus seems that the seeding process encourages an early functional improvement, but it does not lead to a prolonged improvement which "stays ahead" of an unseeded graft. The possibility exists that other factors, such as a cascade of growth factors and the migration of fibroblasts induced by the surgical trauma or the graft itself, exert an influence which outweighs the possible benefits from the seeded cells.

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