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DEVELOPMENT OF A ROTATION DEVICE FOR MICROVASCULAR ENDOTHELIAL CELL SEEDING

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Abstract

A rotation device (RD) specifically designed to achieve sterile endothelial cell (EC) seeding of vascular grafts has been developed. The basic characteristics of the RD include: small dimensions, fully autoclavable components, and perfectly sealed graft holders. These features make it possible to maintain sterility during all the steps of EC seeding. This was documented by negativity of all bacteriological assays performed. Moreover, the RD can simultaneously support three vascular grafts with different lengths (20, 40, and 60 cm) and diameters (4-8 mm). EC seeding is performed in the climatized chamber (37 °C; 5% CO_2) with constant rotation (0.1-3 rpm). The rotation cycle can be completed automatically. The practical efficacy of the RD was investigated by seeding 2 x $10^5/cm^2$ of human microvascular EC on 20 cm length, 4 mm internal diameter (ID) fibronectin-coated polytetrafluoroethylene (ePTFE) grafts for 24 and 48 hours respectively. Further, the effect of a highly viscous plasma expander, i.e., haemagel, on cell retention was also evaluated. Results were not as favorable as expected. However, it should be emphasized that after 48 hours of cell incubation by using the RD, 42% of the initially seeded EC were still present and approximately 15% were fully spread over the graft surface. Moreover, the 10 minute perfusion with haemagel did not decrease the number of adherent microvascular EC.

Key Words: Endothelial seeding, human microvascular endothelial cells, rotation device, scanning electron microscopy, ePTFE graft.

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Introduction

Endothelial cell (EC) seeding was originally postulated as a mechanism for reducing the thrombogenicity of small-diameter synthetic vascular grafts (Herring *et al.*, 1978). However, the clinical applicability of this technique is still under investigation. Most importantly, we have no unequivocal demonstration that the seeded endothelium can significantly improve the long-term patency of small-caliber prosthetic grafts in man (Gordon, 1992).

Conversely, *in vitro* endothelialization of small-diameter synthetic vascular grafts has been definitively documented (Fishlein *et al.*, 1992). Briefly, EC can be harvested from different sources, morphologically and immunophenotypically characterized, as well as seeded on precoated prosthetic graft lumina (Pasquinelli *et al.*, 1990). However, some questions still remain. It is not completely established whether, under these conditions, the seeded endothelium may cover the entire luminal surface of the prosthetic graft. Consequently, a few authors favour an *in vitro* cell culturing approach to achieve shear-stress resistant confluent monolayers prior to implantation (Muller-Glauser *et al.*, 1992). On the other hand, the Vienna group (Fischlein *et al.*, 1992) recently focused on the use of a rotation device (RD) allowing the immediate confluent endothelialization of vascular prostheses of up to 80 cm in length. Despite the hypothetical pivotal role played by RD in graft endothelialization, little has been reported on the basic characteristics of such devices.

We have recently developed an RD specifically designed to perform endothelial seeding under conditions of absolute sterility. This paper describes the design and performance characteristics of this device. The practical efficacy of the RD was investigated by incubating human microvascular EC on fibronectin-coated expanded polytetrafluoroethylene (ePTFE) grafts for 24 and 48 hours, respectively. Further, the effect of a highly viscous plasma expander, i.e., haemagel, on the EC retention was also evaluated.

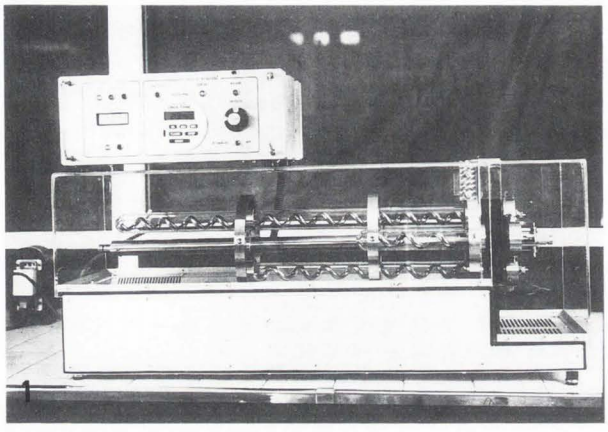


Figure 1. General view of the RD. The main chamber comprising three graft holders of different length and the external control unit are shown.

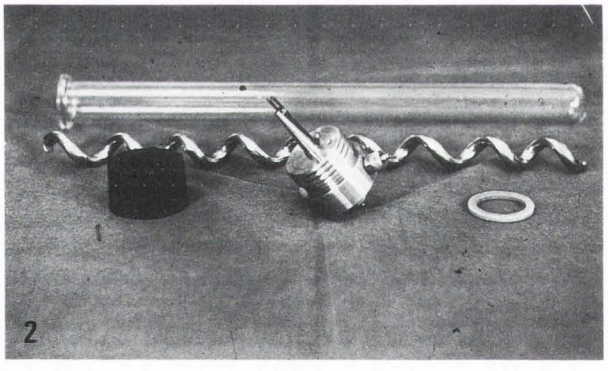


Figure 2. The basic components of the graft holder, i.e., the glass chamber, the steel stainless stabilizer, the O-ring seal, the security cap as well as the collector are illustrated.

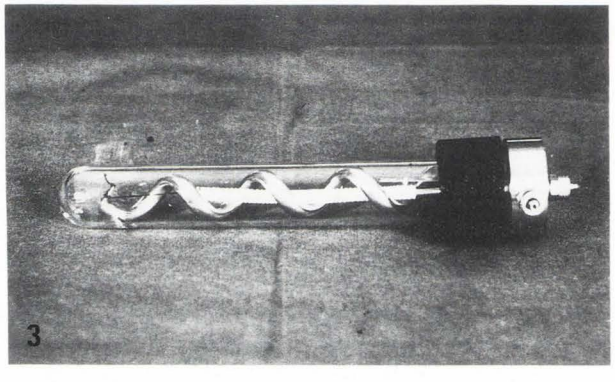


Figure 3. The graft holder of the rotation device with an ePTFE graft aligned on the central axis.

Materials and Methods

Rotation device specifications

The RD is composed of a main chamber comprising three graft holders of different length and an external control unit (Fig. 1). The body of the chamber is closed by a clear acrylic (perspex) cover having an access lateral window. The chamber contains a rotation unit including: **a**) a longitudinally placed stainless steel tube with a short closed perfusion chamber at one side; and **b**) three equally spaced steel rings supporting the graft holders. Rotation is indirectly provided to the steel rings through sliding on cylindrical rollers. This mechanism favours a precise and balanced graft rotation.

The RD displays the following features. Small dimensions (24 x 76 x 30 cm) which fit those of a laminar airflow cabinet. This is useful for performing work under sterile conditions in the operating room. The components are fully autoclavable. The controlled chamber temperature is in the 35-40 °C range. In particular, the RD is fitted with thermoelectric heating and temperature control of $\pm$ 0.5 °C to ensure that the pre-selected temperature is accurately maintained within the chamber. The flexible rotation speed ranges from 0.1 to 3 rpm. The timer can function for runs of up to 24 hours. Both the temperature and the timer can be pre-selected and the temperature carefully monitored on a liquid crystal display. The rotation cycle can thus be completed automatically. An acoustic alarm announces the end of the cycle.

Graft holder specifications

The RD can simultaneously support three grafts of different length, i.e., 20, 40, 60 cm. Individual grafts are inserted into graft holders specifically designed to maintain absolute sterility. As illustrated in Fig. 2, this latter comprises a glass chamber, a coiled graft stabilizer with a terminal ring, an O-ring seal, a security cap, and a collector featuring two separate channels. The coiled stabilizer guarantees the perfect alignment of the graft along the entire length of the holder. Therefore, any kinking of the graft is accurately prevented (Fig. 3). A safe fastening of the graft is obtained by suturing the end of the graft to the terminal ring. The collector displays a conical plug having a terminal flexible pin. This allows the insertion of grafts with diameters between 4-8 mm. Finally, the collector presents suitable adaptor caps as well as plug connectors. Once assembled, the graft holder is laterally loaded in the climatized chamber of the RD. Each holder is secured to the rotation unit by means of a knurled grid.

Flow system

In this case, the holder may serve as a perfusion chamber. Two ports are connected to the exterior via conduits drilled into the collector body, so that solutions may be introduced and removed from the graft through connector tubes in communication with the external perfusion system. Further, two or three grafts can be

simultaneously perfused. In this circumstance, the plug connectors which are located on the external surface of the collector have to be connected to the corresponding plugs of the longitudinal tube.

Method of harvesting human microvascular endothelial cells

Human microvascular endothelial cells were enzymatically harvested as previously described (Curti *et al.*, 1989). Approximately 15 g of subcutaneous fat was sterilely removed by simple excision from the abdominal walls from 8 patients undergoing surgery for abdominal aneurysms. To avoid undesirable toxicity of glove powder, starch-free gloves were used (Sharefkin *et al.*, 1986). The fat tissue was minced in a Petri dish containing cold phosphate buffered saline (PBS) under a laminar airflow cabinet. The minced fat was transferred into a sterile Erlenmeyer flask for collagenase incubation. The enzyme (Type V Collagenase, Sigma, St. Louis, MO, USA) was prepared by dissolving 100 mg of collagenase (activity level 600 U/mg) in 50 ml of PBS. Fifty ml of collagenase solution were warmed to 37 °C prior to use. The flask containing the minced fat was incubated for 30 minutes at 37 °C with constant agitation in a shaker water bath. Following incubation, the top layer consisting of floating adipocytes was discharged whereas the remaining suspension was filtered through a 400-μm nylon filter to remove undesired remnants of connective tissue. The suspension was then centrifuged at 200 g for 10 minutes. The resulting pellet containing microvascular cells was then resuspended in sterile PBS containing 0.1% bovine serum albumin (BSA) and repeatedly pipetted to disperse the cell pellet. After being centrifuged again, the pellet was resuspended in 2 ml of PBS-BSA and gently layered on 8 ml of sterile 45% Percoll (Sigma) in PBS (280 mOSM) and centrifuged at 1500 g for 20 minutes at room temperature. Microvascular cells were recovered in a milky-white layer at the top of the Percoll cushion. After being repeatedly washed in PBS-BSA, the microvascular cells were suspended in sterile Medium M199 (Gibco, Grand Island, New York, USA) containing 20% heat-inactivated fetal calf serum (Gibco), 25.000 U/ml heparin (Sigma), 3 mg/ml endothelial cell derived growth factor (Sigma), 20 mM L-glutamine (Gibco), 100 U/ml penicillin (Flow, Costa Mesa, CA, USA), 100 μg/ml streptomycin (Flow), and 20 mM HEPES (Gibco) (complete medium). Cells were counted in a haemocytometer whereas viability was determined by trypan blue exclusion.

Method of graft seeding

20 cm length, 4 mm internal diameter (ID) ePTFE grafts (W.L. Gore & Associates, Inc., Flagstaff, AZ, USA) were inserted in the graft holder of the RD. Prior to seeding, ePTFE grafts were perfused with 10 μg/cm human fibronectin (Sigma) for 30 minutes at 37 °C. Approximately 5 ml of seeding inoculum containing an average of 1.1 x 10^6 cells/ml was injected from a syringe using very low pressure into the lumen of the graft until the culture medium could be seen bubbling on the outer surfaces of the graft. Consequently, ePTFE grafts were seeded with 2 x 10^5 cells per cm^2 surface area. The graft containing the inoculum was then rotated at 0.3 rpm in the climatized (37 °C, 5% CO_2) RD for 24 and 48 hours, respectively. After the rotation cycle was ended, the inoculum was discharged and the graft lumen gently perfused with complete Medium 199. Aliquots of the recovered inoculum were assayed for eventual aerobic and anaerobic bacterial contamination. The entire graft was then perfused with a pre-heated 2.5% glutaraldehyde buffered solution. The graft was removed from the holder and routinely processed for scanning electron microscopy (SEM).

Method of perfusion with haemagel

In a few cases, following 48 hours of EC seeding, the external circuit of RD was activated and approximately 100 ml of haemagel solution (Behring, Scoppito, Italy) was pumped (35 ml/min) into the system by using a roller pump for 10 minutes. To achieve a steady flow, a funnel was used, causing a steady flow rate by means of gravity. A corresponding wall shear rate of 800 s^{-1} was calculated according to Muggli's formula (Muggli *et al.*, 1980). After several washes with complete medium, the specimens were fixed with glutaraldehyde and processed for SEM.

Method for scanning electron microscopy (SEM)

After glutaraldehyde fixation, the grafts were serially cut into 1 x 1 cm segments, post-fixed with 1% osmium tetroxide for 10 minutes, repeatedly washed in distilled water, dehydrated in a series of graded alcohols and critical point dried. The samples were mounted with conductive silver glue on aluminium stubs and coated with a 10 nm thick gold film in a sputtering device. he mounted specimens were observed in a Philips 505 scanning electron microscope operated at intermediate accelerating voltages (15-21 kV).

Results

Following 24 hours of cell incubation on fibronectin coated ePTFE grafts, approximately 80% of microvascular cells were retained (1.6 x 10^5 cells/cm^2). Surprisingly, most EC were rounded in shape (Fig. 4) and unevenly distributed along the entire graft length. SEM disclosed early cell contacts with the surface. Initial spreading was observed in correspondence with the nodes of the ePTFE material (Fig. 5). The spread cells apparently extended towards the internodal areas.

After 48 hours of incubation the number of adherent EC had decreased further (8.4 x 10^4 cells/cm^2) and only 42% of the initially seeded microvascular cells were apparently retained. The EC were more completely flattened and spread on the graft surface (Fig. 6). However, approximately 60% of the adherent cells were still rounded. Many of the latter showed degenerative changes including surface blebbing and plasmalemmal holes (Fig. 7).

SEM of the grafts following 10 minutes haemagel perfusion still showed an EC lining. The number of adherent cells was approximately the same as the corresponding grafts without haemagel perfusion (9.6×10^4 cells/cm^2). However, in this case, most of the spread cells returned towards a rounded morphology and focally contracted into groups (Fig.8).

Bacteriological tests were negative.

Discussion

RD is believed to establish confluent monolayers of EC on fibrin glue coated vascular grafts (Fischlein *et al.*, 1992). However, to date investigators using RD have not sufficiently reported the characteristics concerning such devices (Fischlein *et al.*, 1992; Fasol *et al.*, 1987; Prendiville *et al.*, 1991).

This is the first paper providing the design and the performance characteristics of an originally developed RD. The RD presented here was designed to perform EC seeding under conditions of absolute sterility. This latter feature is particularly crucial when implants of endothelial seeded synthetic vascular grafts have to be performed in humans. As documented by means of bacteriological assays, the present study clearly demonstrated that sterile EC seeding of vascular grafts can be achieved by using an RD.

In order to establish the practical efficacy of the RD, microvascular EC (Jarrell *et al.*, 1984) were inoculated into 20 cm length, 4 mm ID, fibronectin-coated ePTFE grafts, by using the climatized (37°C, 5% CO_2) RD. Following 24 hours of microvascular cell incubation, 80% of the initially seeded EC were retained. SEM investigation showed mostly rounded EC which were unevenly distributed along the entire graft surface. As expected, this feature was less apparent after 48 hours of cell incubation. The pattern of cell alignment seemed to reflect the site of the initial cell attachment on the graft lumina. This finding was almost disappointing and contrasts with that recently reported by the Vienna group (Fischlein *et al.*, 1992). The uneven distribution of the seeded cells is not apparently related to the performance of the rotation unit nor can it be imputable to uneven fibronectin coatings of the graft lumen. In fact, cell incubation as well as fibronectin graft pre-coating were carried out by using constant rotation (0.3 rpm). Therefore, a more homogeneous cell distribution could be expected by performing even the initial cell inoculum under rotation.

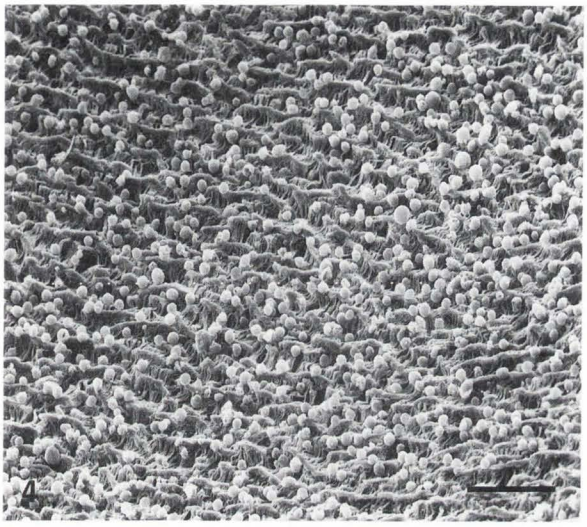
After 48 hours of cell incubation, we found 42% of the initially seeded cells displaying a more orderly cell distribution. Further, many cells were completely spread over the graft surface. The fact that in our experimental model, only a small percentage of the initially seeded cells (15%) spread over the graft surface, may be related to several factors. Firstly, functional differences between human microvascular cells and enzymatically harvested venous EC should not be underestimated. Alternatively, the adverse effect of Percoll separation on microvascular cell adhesion and growth in culture could also be implicated as recently hypothesized by Meerbaum *et al.* (1992). This feature is further suggested by the SEM demonstration of degenerating round EC at 48 hours. Then, shear stress could promote both cell alignment and spreading as suggested by Pratt *et al* (1988). Multiplication of harvested EC under tissue culture conditions could also modify some functional characteristics of the EC. This could at least partially explain the data provided by the Vienna group (Fischlein *et al.*, 1992). It further remains to be established whether 72 hours of cell incubation on ePTFE grafts can significantly improve the confluency of the seeded endothelium (Prendiville *et al.*, 1991). Finally, it should also be mentioned that only 55.8% of the total microvascular cells stained positive for Factor VIII (Meerbaum *et al.*, 1992). However, the present investigation should alert investigators of the pros and cons of using microvascular seeded vascular grafts for clinical trials.

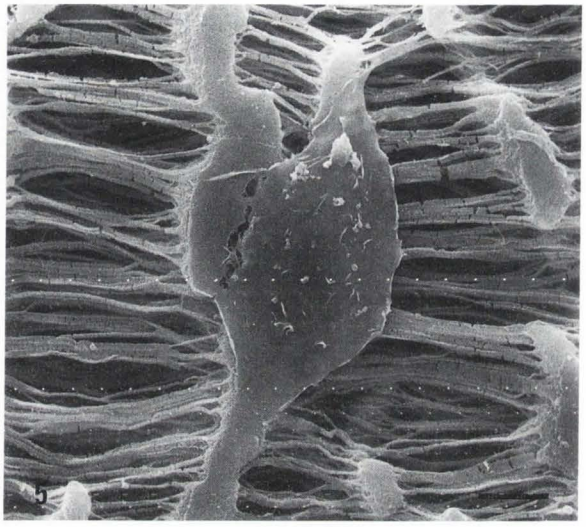
The effect of haemagel on cell retention was also investigated. The use of this high viscous plasma expander does not decrease the number of the seeded cells. However, most of EC were rounded. Consequently, we do not know whether a longer perfusion time could drastically affect the number of the adherent microvascular EC. Rosenmann *et al.* (1985) clearly demonstrated that at 24 hours only 17% of all seeded cells were still present on ePTFE grafts after flow restoration. It is therefore possible to expect similar findings also in our experimental model.

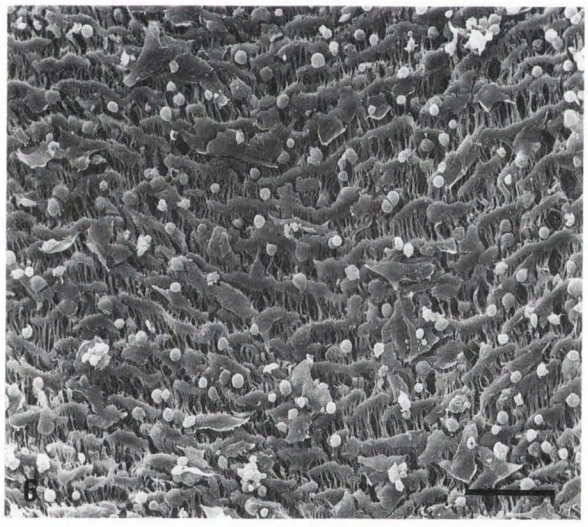
In conclusion, the design as well as the performance characteristics of an RD are presented here. This device allows the performance of endothelial seeding under conditions of absolute sterility. However, confluent cell monolayers were not achieved by seeding microvascular EC on fibronectin coated ePTFE grafts. This unexpected finding may be due to different factors including eventual differences in functional characteristics of microvascular EC. However, it should be emphasized that after 48 hours of cell incubation by using the RD, 42% of the initially seeded EC were still present and approximately 15% were fully spread over the graft surface. Moreover, a 10 minute perfusion with a highly viscous solution, namely haemagel, did not decrease the number of adherent cells. In future, progress in modifying synthetic vascular grafts as those proposed by the Aachen group (Kirkpatrick *et al.*, 1991), as well as suitable alternatives to molecules (fibronectin and fibrin glue) which are currently used to precoat vascular materials, are needed to perform effective implants in humans.

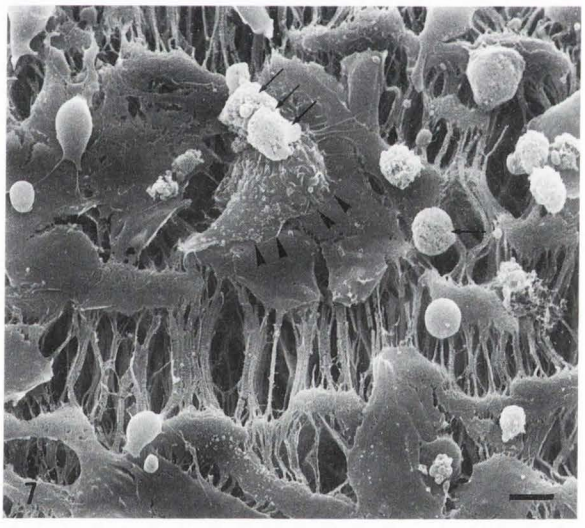
Acknowledgments

The skillful assistance of Mr. S. Domanin and Mr. A. Silvestrini is greatly appreciated.









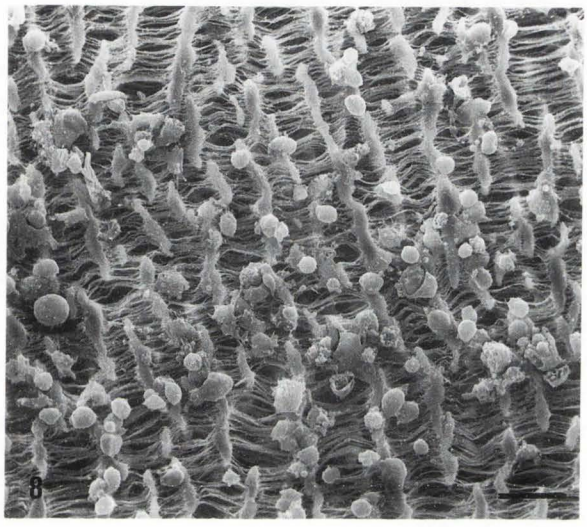


Figure 4. 24 hours of microvascular EC incubation on fibronectin-coated ePTFE grafts. SEM view of adherent EC. Most cells are rounded in shape. Bar = 50 μm.

Figure 5. 24 hours of microvascular EC incubation on fibronectin-coated ePTFE grafts. A spread cell is observed in correspondence with the nodes of the ePTFE material. Bar = 5 μm.

Figure 6. 48 hours of microvascular EC incubation on fibronectin-coated ePTFE grafts. SEM shows an increased number of fully spread microvascular cells. Bar = 50 μm.

Figure 7. 48 hours of microvascular EC incubation on fibronectin-coated ePTFE grafts. Some of the rounded cells show degenerative features (arrows). A leukocyte is observed on the spread microvascular EC (arrowheads). Bar = 10 μm.

Figure 8. 48 hours of microvascular EC incubation on fibronectin-coated ePTFE grafts. After 10 minutes of haemagel perfusion EC are still present on the graft surface. Most of these cells are rounded in shape. Bar = 20 μm.

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Discussion with Reviewers

E.W.Y. Koo: It would be helpful to include a control where under sterile conditions, seeding of the same number of endothelial cells (EC) occurred in the absence of rotation.

Authors: This experimental condition was carried out; after 48 hours of cell incubation, we observed a high density layer of EC with a rounded appearance. Very few spread cells were found. EC crowded the lower concavity of the tubular graft. As a consequence, we seriously considered that the presence of a rotation cycle is indispensable to facilitate homogeneous cell distribution and spreading on the tubular graft lumina.

E.W.Y. Koo: Many of the seeded EC showed a rounded morphology, even after 48 hours following seeding. Could some of these cells be undergoing cell replication or are these EC able to proliferate at all?

Authors: We have no data suggesting that the seeded EC could eventually proliferate during incubation. However, in our experience, standard cultures of microvascular EC significantly differ from those obtained from venous EC (both umbilical and saphenous veins). Apart from the morphological appearance, microvascular cells display a delayed and low proliferation rate as well as low prostacyclin synthesis and a high t-PA/PAI-1 ratio.

E.W.Y. Koo: Have the authors used substrates other than fibronectin?

Authors: No, we have not used other substrates as yet.

E.W.Y. Koo: What criteria are used to call the cell a leukocyte in Fig. 7?

Authors: Basically, we used the following criteria: characteristic of spreading, shape, and presence of numerous ruffles on the cell surface.

E.W.Y. Koo: Why are the adherent EC undergoing degenerative changes?

Authors: We feel that the presence of toxic substances in commonly used laboratory equipments and/or chemicals, e.g., endotoxins possibly contaminating BSA stocks as suggested by R.M.K.W. Lee (see below), may be responsible for these degenerative changes. However, the effects of residual monocyte/macrophages should not be underestimated.

R.M.K.W. Lee: You mentioned that 0.1% BSA was used to resuspend the cells. Most BSA contained pyrogens, and one of which is endotoxin. Did you test your BSA? This is important for this type of work.
Authors: No, we did not test the BSA. This could at least in part explain why we observed degenerating cells during incubation. Thank you for this suggestion.

R.M.K.W. Lee: You have quoted a recent paper by Meerbaum *et al.* (1992), that 56% of the microvascular cells were positive to factor VIII, probably meaning that these might be EC. What is the composition of the microvascular cells you have used? This may explain the differences in the cell attachment as compared with results when pure EC were used.
Authors: The primary cell isolates were investigated by transmission electron microscopy (TEM) and immunohistochemistry (immunofluorescence, APAAP and protein A-gold plus silver enhancement). With TEM, microvascular cells were ultrastructurally homogeneous. Interestingly, we found Weibel-Palade bodies only in the cytoplasm of cell clumps. Immunohistochemically, 5% of cells expressed factor VIII RA, 18% stained for CD31 (EC lineage marker), 1-2% expressed monocyte/macrophage specific antigens (150.95 protein and MAC 387). Immuno-staining for desmin and S-100 protein was almost negative. Smooth muscle-specific actin and GP IIb/IIIa were faintly expressed. Remarkably, endothelial-specific antigens were mostly found in cell clumps. We believe that microvascular cell isolates mostly consist of EC. However, it can be suggested that as EC lose intercellular contacts, the isolated cells undergo a phenotypic shift towards mesenchymal cells able to further differentiate along the endothelial lineage.

R.M.K.W. Lee: What was the percent viability of the cells according to your trypan blue test? The rounded cells 24 hours after seeding could be dead cells.
Authors: In the primary cell isolate it was better than 90%. Consequently, we do not think that the rounded cells could be dead cells.

R.M.K.W. Lee: How did you establish that injecting 1.1×10^6 cells/ml would give you a seeding density of 2×10^5 cells/cm^2? This is important because your percent attached cells, i.e., 80%, was based on this calculation. What method did you use to estimate your cell density using SEM? How many experiments were carried out, and what was the standard error?

Authors: Firstly we calculated the total area to be seeded with cells. In the case of 20 cm long, 4 mm in diameter ePTFE grafts, the estimated area was 25.12 cm^2. Since we injected 5 ml of complete medium containing approximately 1.1×10^6 cells/ml the corresponding seeding density was 2×10^5 cells/cm^2.

Adherent cells were counted on low magnification scanning electron micrographs (300x final magnification). The micrographs were representative of 10 segments of the seeded graft. Counting was performed by a student who was not informed about the finalities of the work. A grid containing 8 squares measuring 0.1 mm was used. The total number of cells as well as relative number of rounded and spread cells were measured. A mean value was finally obtained. Eight experiments were carried out. The standard error was not calculated. Although counting of seeded EC by SEM does not represent an accurate method for quantifying cell numbers, it allows a "rough" estimation of cell adhesion on vascular substrates. Further, as recently reported by M.J.T. Visser *et al.* [Accuracy of quantification of seeded endothelial cells. In: Applied Cardiovascular Biology 1990-1991, Vol. 2. Zilla P, Fasol R, Callow A (eds.). Karger, Basel, 1992, pp. 97-99], counting by scanning electron microscopy has the same low accuracy as other standard methods have, e.g., enzymatic removal of cells and cell counting in a haemocytometer, or counting of hematoxylin-stained cells by light microscopy with an ocular microgrid. Further, counting by scanning electron microscopy allows a clear distinction between variously shaped cells and displays a low inter-observer variability.

R.M.K.W. Lee: To be of any practical use, it is essential to establish that a continuous cell lining will be formed in these grafts at some juncture. Has this been shown by others?
Authors: In the experimental models this has been extensively demonstrated. In humans, we are still lacking any definitive proof.

M. Sigot-Luizard: Did you check factor VIII synthesis on the remaining cells spread on the prosthesis? It would be interesting to know how many functional EC are still remaining on the prosthesis at the end of the rotating experiment.
Authors: We did not try to recover EC at the end of the rotation cycle. Therefore, the factor VIII synthesis was not evaluated in the present study.