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Coating PTFE vascular prostheses with a fibroblastic matrix improves cell retention when subjected to blood flow

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Abstract: An investigation was made into the effect of blood flow on endothelial cells (EC) and mesothelial cells (MC) seeded on a vascular expanded polytetrafluoroethylene (ePTFE) prosthesis coated with a fibroblastic matrix. Endothelial cells were obtained from the external jugular vein and MC from the omentum. To test the performance of prostheses, a custom designed, femoral "*ex vivo*" circuit was developed in mongrel dogs. Four study groups were established: a control group, A_1, where prostheses were uncoated and seeded with EC; a second control group, A_2, where prostheses were uncoated and seeded with MC; group B_1 where prostheses were coated with a fibroblastic matrix and seeded with EC; and group B_2 where coated prostheses were seeded with MC. All cells were labeled with ^{111}Indium oxine (10 mCi/mL) before seeding. After the seeded cells had formed a monolayer on the ePTFE prostheses (which took approximately 24 h) the prostheses were placed in the "*ex vivo*" circuit. The rates of blood flow to which prostheses were exposed were measured at the point of inflow (117.5 ± 12.50 mL/min, mean ± SD) and outflow (72.6 ± 14.3 mL/ min). MC showed a greater baseline radionuclide uptake than did EC. The cells of groups B_1 and B_2 adhered sufficiently to the fibroblastic matrix and covered enough of the prosthetic surface to be positioned in the "*ex vivo*" circuit (76.90 ± 8.24% surface covered in EC-seeded prostheses and 71.65 ± 6.23% in MC-seeded prostheses). After exposure to blood flow the quantity of radionuclide-labeled cells and the prosthetic surface covered by them were greatly reduced though the fibroblast-coated prostheses showed greater cell retention. © 1998 John Wiley & Sons, Inc. *J Biomed Mater Res,* **39,** 32–39, 1998.

Key words: endothelial cells; mesothelial cells; cell seeding; PTFE; blood flow

INTRODUCTION

It is believed that coating the luminal surface of small-caliber vascular prostheses with a layer of cells improves clinical results after implantation. For this purpose, endothelial cells (EC) from venous sources have been widely used.[1–4]

The use of human veins, however, is not always practical since autologous veins are still the best option for "by-pass," or vascular replacement, surgery. An answer to this supply problem could be the use of mesothelial cells (MC) obtained from the omentum. MC have been shown to give good results with respect to availability, ease of culture, and ability to form a confluent monolayer on the biomaterial similar to that formed by EC.[5] The true identity of omental cells is debatable. Some authors suggest that they are of endothelial origin[6,7] since they come from microvessels; others believe them to be of mesothelial origin;[5,8] and a third group suggests that currently available criteria are insufficient to permit their classification as either endothelial or mesothelial cells.[9]

In a previous study[5] omental MC were found to react with both EC antibodies and antibodies specific to smooth-muscle cells (anti-a actin and anti-total actin). It also was observed that MC seeded on expanded polytetrafluoroethylene (ePTFE) prostheses showed similar behavior to EC and, as reported by other authors,[5,8] optimal prostacyclin production.

Good cellular adherence to the prosthetic material and cell retention are important factors to be considered when using a biomaterial in vascular surgery.

Some authors have shown that the endothelial function of such prostheses is better when the layer on which the endothelium rests resembles the vascular medial layer.[10] This has led to studies comparing several coatings and to the use of proteins to improve cellular adhesion.[11,12] Such studies have produced good results with respect to initial cell adherence and to the formation of a confluent monolayer of cells on the biomaterial, but little success has been attained

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with respect to the stability of cell coverage after exposure to blood flow.

In an effort to improve cell retention on the biomaterial the present study investigates the coating of the prosthesis with a matrix of fibroblasts prior to seeding. In a previous study in which the prostheses were not exposed to blood flow, significantly better results were obtained with biomaterials coated with a fibroblastic matrix than with those that were treated only with fibronectin.[13]

These findings led to the present study in which the effect of blood flow on fibroblast-coated prostheses seeded with EC and MC were investigated. A dynamic blood flow system was developed in the dog in which the protheses formed part of an "*ex vivo*" circuit between the femoral arteries and the right atrium. ECseeded and MC-seeded vascular ePTFE prostheses without fibroblastic matrices were used as controls.

MATERIAL AND METHODS

Experimental animals

Twelve mongrel dogs weighing from 12 to 14 kg were used for the study. Fragments of the external jugular vein, omentum, and skin were obtained from each animal for cell culture. Before implantation of the seeded prostheses, anesthesia was induced with sodium thiopental (0.5–1 mg/kg) and maintained with a combination of halothane (1.5–2%), oxygen, and nitrous oxide. (Care of the animals complied with the rules stipulated by the Principles of Laboratory Animals produced by the National Society for Medical Research and the National Academy of Sciences, respectively).

Cell cultures for seeding

Endothelial cells (EC)

Endothelial cells were obtained from the external jugular vein of each dog. The vein repeatedly was flushed with Minimal Essential Medium (MEM, GIBCO BRL, Izasa, Madrid) under sterile conditions to remove red blood cells. It then was filled to distention with a 0.1% CLS collagenase type I solution in 15 mmol/L $CaCl_2$ (Lorne Laboratories Ltd., Twyford, U.K.) following the technique described by Budd et al.[14] The resulting cell suspension was centrifuged at 200 g for 7 min and the supernatant discarded. The cell pellet was resuspended in complete M-199 medium consisting of standard M-199 (GIBCO BRL, Izasa, Madrid), 20% fetal bovine serum (GIBCO BRL, Izasa, Madrid), 90 mg/ mL heparin-porcine intestinal mucosa (SIGMA, Madrid), 2 mmol L-glutamine (GIBCO BRL, Izasa, Madrid), 20 mg/ mL endothelial growth factor (SIGMA, Madrid, Spain), 20 nmol Hepes buffer (GIBCO BRL, Izasa, Madrid), and 10,000 IU/ mL penicillin plus 10,000 mg/mL streptomycin (GIBCO BRL, Izasa, Madrid). A small aliquot was taken for cell counting and the remainder of the cell suspension was seeded in 25 cm^2 culture flasks (NUNC-INTERMED, Lab-Clinics, Barcelona).

Mesothelial cells (MC)

Fragments of omentum (20–50 g) were obtained from the animals by minilaparotomy. The omentum was submerged in MEM supplemented with an antibiotic/antimycotic solution of 10,000 IU/mL penicillin, 10,000 mg/mL streptomycin, and 25 mg/mL amphotericin B/Fungizone (GIBCO BRL, Izasa, Madrid). It then was washed several times in MEM and the cells extracted by CLS type I collagenase digestion (0.1% in 15 m*M* $CaCl_2$) for 20 min in a shaker bath maintained at 37°C.

Following enzyme digestion, the cell suspension was placed in a 50 mL conical centrifuge tube (NUNCINTERMED, LabClinics, Barcelona) and centrifuged for 7 min at 200 g. The supernatant was discarded and the pellet resuspended in 5 mL of M-199 supplemented with 20% fetal bovine serum (GIBCO BRL, Izasa, Madrid), 10 m*M* Hepes, 10,000 IU/mL penicillin/10,000 mg/mL streptomycin, 2 m*M* L-glutamine (GIBCO BRL, Izasa, Madrid), and 90 mg/ mL sodium heparin (Roche, Madrid). The cell suspension was placed in a 25-cm^2 culture flask and incubated at 37°C in a 5% CO_2 atmosphere.

Fibroblasts

A small skin specimen (1 cm × 0.5 cm) was obtained from each animal and submerged in MEM. Explants were placed in culture flasks containing 5 mL of McCoy's complete medium (GIBCO BRL, Izasa, Madrid) and incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced every 2 days until a confluent monolayer had formed on the walls of the culture flask.

Cell subcultures

Cells were separated from the flask after the monolayer had formed using a solution of Trypsin-EDTA IX (GIBCO BRL, Izasa, Madrid), which was allowed to act for five min at 37°C. Enzyme action was interrupted by complete M-199. The resulting cell suspension was divided into culture flasks at a ratio of 1:2.

Cell counting and viability

The number of viable EC, MC, and fibroblasts was determined by trypan blue exclusion and by counting in a Neumbauer chamber.

Cell identification

Cells were identified by an immunohistochemical technique using anti-CD 34 QBEND10 (SEROTEC, Oxford, England),

antivimentin (BIOMEDA, Madrid), antidesmin, anti-a actin and anti-total actin (all courtesy of Professor Gabbiani, Geneva, Switzerland).

Prostacyclin production

Prostacyclin levels were determined by measurement of the concentration of a stable intermediate of the prostacyclin synthesis pathway, 6-ketoprostaglandin F_{1a}, using a radioimmunoassay kit (AMERSHAM IBERICA S.A., Madrid).

Cell labeling with ^{111}In oxine

Before seeding onto prostheses, EC and MC were labeled with ^{111}In oxine (1 mCi/mL) (NUCLEAR IBERICA S.A., Madrid) following the technique described by Sharefkin et al.[15] ^{111}In oxine (10 mCi/mL) was added to the cells in serum-free M-199 and the suspension incubated for 15 min at room temperature and centrifuged at 200 g for 7 min. The supernatant was removed to eliminate excess radionuclides and the cell pellet washed with PBS. The cells were resuspended in complete M-199 before seeding onto the fibroblastic matrix.

Since seeded cells may undergo considerable spontaneous radionuclide loss, particularly after exposure to blood flow,[16] the radionuclide activity in this study was measured immediately before positioning the prosthesis in the "*ex vivo*" circuit, and results were expressed in terms of loss of radionuclide rather than in loss of cells.

Cell seeding on ePTFE

Cells were seeded onto expanded polytetrafluoroethylene (ePTFE) vascular grafts (W.L. Gore & Associates, INC., Flagstaff, AZ). The grafts were 6 cm × 4 mm in internal diameter and 22–30 mm in internodal distance. They were cannulated at both ends and placed in glass containers.

Fibroblast seeding

Fibroblasts for seeding were separated from the first subculture and centrifuged (200 g) following trypsin treatment and inactivated in McCoy's complete medium. The resulting cell pellet was resuspended in the medium to provide cells for seeding. The prostheses were treated with a fibronectin solution (20 mg/mL) for 1 h to facilitate cell adhesion.[17]

Prostheses were seeded by flooding with a fibroblast suspension of 2×10^5 cells/mL and incubated at 37°C for 15 min to allow cellular adhesion to the surface of the ePTFE. Following the removal of excess medium, prostheses were turned 90 degrees and reseeded four times according to the procedure described above. After seeding, the prostheses were incubated at 37°C for 24 h with gentle rotation (10 rph). Once a monolayer had formed (about 24 h after seeding), the cells were fixed to the graft surface by treatment with 5% glycerol for 5 min.[18] After fixation, the surface was washed with MEM to completely eliminate the fixative.

EC/MC seeding onto coated and uncoated prostheses

The MC and EC obtained from the first subculture were centrifuged at 200 g for 7 min, then resuspended in M-199 and seeded on the coated and uncoated ePTFE at a density of $1.5–2 \times 10^5$ cells/mL. The seeding procedure was the same as described above for fibroblasts.

Ex vivo circuit

An in-house femoro-atrial "*ex vivo*" circuit was set up in each dog (Fig. 1) by cannulating both femoral arteries and the right atrial appendage, accessed by medial sternotomy. A polyethylene tube (4 mm inner diameter) was used to connect each femoral artery to a prosthesis, and the two prostheses were connected to polyethylene tubes that were joined by a Y connection to the right atrium. Sodium heparin (3000 IU) was administered to each animal prior to surgical intervention.

Femoro-atrial blood flow was maintained for 1 h. The rate of blood flow was measured using sterile tubing flow sensors (model 5c) (Transonic Systems, Inc. Ithaca, NY) placed at the points of inflow and outflow of the prosthesis.

Scanning electron microscopy (SEM)

The prostheses were removed from the "*ex vivo*" circuit and fixed in 3% glutaraldehyde for 2 h, introduced in Millonig's buffer (pH 7.3), and dehydrated in a graded acetone series, reaching critical point of dehydration in a CO_2 Polaron E-3000. Samples were metallized with gold-palladium and examined using a Zeiss 950 DSM scanning electron microscope.

Measurement of covered prosthetic surface

In order to quantify seeding of the prosthetic surface, the covered area was estimated by planimetric analysis using a Microm Analyzer.[18]

Experimental design

The following study groups were established:

- Group A_1 ($n = 6$): EC seeded directly on uncoated ePTFE prostheses
- Group A_2 ($n = 6$): MC seeded directly on uncoated ePTFE prostheses
- Group B_1 ($n = 6$): EC seeded on prostheses coated with a fibroblastic matrix
- Group B_2 ($n = 6$): MC seeded on prostheses coated with a fibroblastic matrix

We did not consider the study of a third control group with fibroblastic matrix without seeding because this organic matrix shows a strong adherent surface, which gives rise to a great adhesion of platelets. This would make difficult a comparative analysis of the surface coated by the fibroblastic matrix under normal blood flow conditions.

The seeded prostheses were positioned in the "*ex vivo*" circuits described above and exposed to blood flow. In six dogs the circuit contained prostheses from groups A_1 and B_1, and in the remaining animals, prostheses from groups A_2 and B_2 were introduced. Radionuclide activity in each prosthesis was measured using a gamma counter (Ram DA-3, Rotam, Industries Ltd; Probe, PM-10, Rotem Industries Ltd) at time 1 (onset of blood flow in the circuit) and at 10 min intervals for 1 h. In order to measure the amount of radioactivity remaining on the prosthetic surface after each experiment, the circuit was closed (by closing the three valves), and the lumen of the prosthesis was washed with 10 mL of physiological saline. In this manner, the amount of radioactive isotope remaining on the prosthetic surface was determined since any ^{111}In oxine circulating in the blood would have been removed by the rinsing process.

The prostheses were removed from the circuit after 1 h and washed twice with saline solution to remove any blood. Radionuclide activity in the prosthesis also was determined after removal from the circuit.

Statistical study

The Mann-Whitney *U* test was used to compare ^{111}In oxine retention between groups. Differences in the remaining cell-covered surface areas were analyzed using the Student– Newman–Keul test.

RESULTS

EC and MC were clearly positive for anti-CD 34 (QBEND10) and antifactor VIII antibodies. Fibroblasts proved positive for antivimentin antibodies. MC also showed positive reactions to antidesmin, antialpha actin and antitotal actin antibodies. Both EC and MC contained high levels of prostacyclin in culture (> 5 ng/mL), but no prostacyclin activity was detected in the fibroblasts.[18]

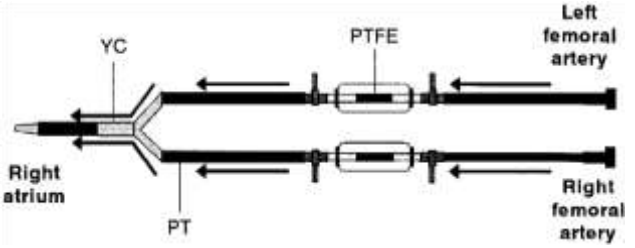

Figure 1. Femoro-atrial "*ex vivo*" circuit. PT: polyethylene tube; PTFE: polytetrafluoroethylene prostheses; YC: Yconnection; arrows indicate direction of blood flow.

value.

Group A_2 (n = 6)

The fibroblasts examined on the ePTFE were rounded with numerous spiky prolongations that connected the cells to form a typical cellular reticulum. They formed a stable monolayer on the surfaces of the prostheses 18 to 24 h after seeding.

EC and MC formed a confluent monolayer on the prostheses, showing rounded cells at different stages of division (Fig. 2).

The effectiveness of cell seeding and retention was estimated by comparison of radioactivity before and after exposure to blood flow. The average rate of blood flow in the animals was 117 ± 12.5 mL/min at inflow to the prosthesis and 72.6 ± 14.3 mL/min at outflow. This reduction in rate was due to the resistance of the biomaterial to blood flow.

Groups A_1 and A_2 (controls)

Group A_1 (n = 6)

In the ePTFE prostheses seeded directly (no fibroblastic matrix) with ^{111}In oxine-labeled EC and exposed to blood flow, most of the radionuclide activity had disappeared at timepoint 1 (onset of blood flow in the "*ex vivo*" circuit), leaving only 13.59% of the baseline activity. After 10 min of blood flow, radionuclide activity had decreased to 12.07% of the baseline value. Measurements taken thereafter (10 min intervals for 1 h) remained fairly stable, reaching 9.33% of baseline

The effect of exposure to blood flow on the uncoated ePTFE prostheses seeded with ^{111}In oxine-labeled MC was similar to that observed for EC. Radionuclide activity ranged from 12.67% of baseline value at timepoint 1 to 7.67% 1 h after exposure to blood flow (Fig. 3). No statistically significant difference in radionuclide activity was observed between groups A_1 and A_2.

Groups B_1 and B_2

Group B_1 (n = 6)

In the ePTFE prostheses coated with a fibroblastic matrix and seeded with ^{111}In oxine-labeled EC, radionuclide activity was 25.03% of baseline value at onset of blood flow, 23.87% at 10 min, and 20.28% at 1 h.

The level of radionuclide activity after exposure to blood flow was significantly higher ($p < 0.001$) in ECseeded

ePTFE prostheses coated with a fibroblastic matrix than in EC seeded on uncoated prostheses [Fig. 4(a)].

Group B_2 ($n = 6$)

The effect of exposure to blood flow on radionuclide activity in coated prostheses seeded with MC was sig-

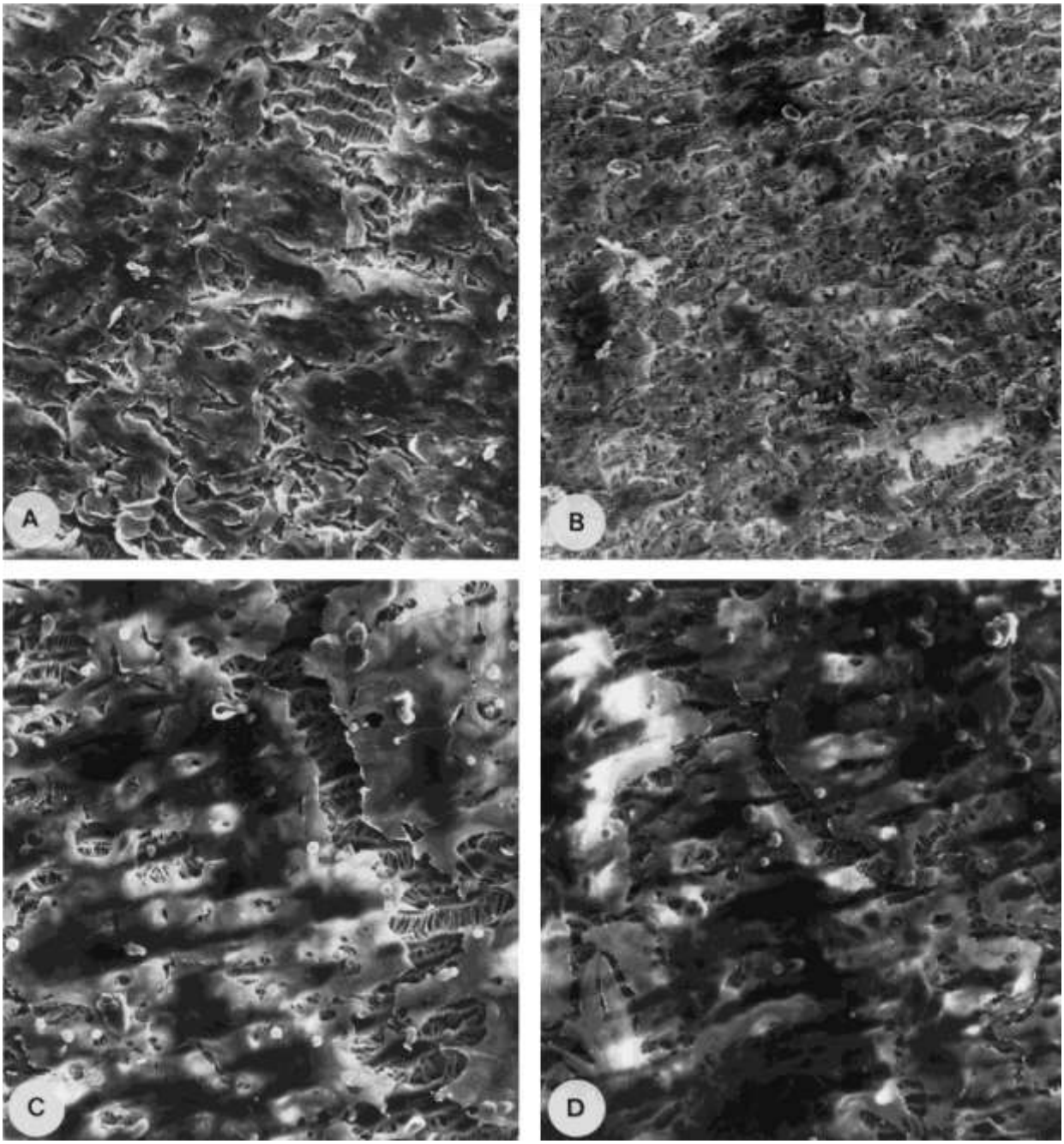

Figure 2. Cell seeding on ePTFE before exposure to blood flow: (a) Group A_1—EC/uncoated prosthesis 100 ×; (b) Group A_2—MC/uncoated prosthesis 100 ×; (c) Group B_1—EC/coated prosthesis 200 ×; (d) Group B_2—MC/coated prosthesis 200 ×.

nificantly different ($p < 0.001$) than the effect on the control group. Radionuclide activity in the coated prostheses decreased from 26.9% at timepoint 1 to 21.01% 1 h after exposure to blood flow [Fig. 4(b)].

The results indicate that radionuclide activity in EC and MC seeded on coated prostheses did not differ significantly after exposure to blood flow.

Scanning electron microscopy after exposure to blood flow

SEM confirmed the results obtained above. After blood flow the surface of the uncoated prostheses showed only a few areas of stretched cells (both EC and MC) that maintained the morphology observed before exposure to blood flow.

SEM demonstrated that when the coated prostheses were exposed to blood flow, the EC and MC remained in the areas in which the fibroblastic matrix offered resistance to blood flow. In these areas, the cells conserved the same flattened morphology seen before exposure to blood flow.

Measurement of covered prosthetic surface

The covered area of the prostheses was estimated before and after onset of blood flow in the circuit:

Before blood flow

The success of seeding (measured as the surface area of the prosthesis covered after 24 h) ranged from

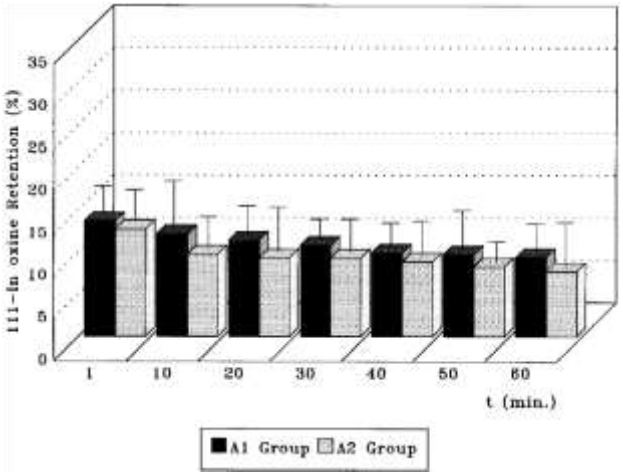

Figure 3. Retention of radionuclide activity on the of MC seeded on uncoated ePTFE after exposure to blood flow. This percentage refers to the number of cpm (counts per minute, y axis) at a given time (t) in relation to the initial time (0, before circuit, x axis). There were no statistically significant differences between control groups A_1 and A_2.

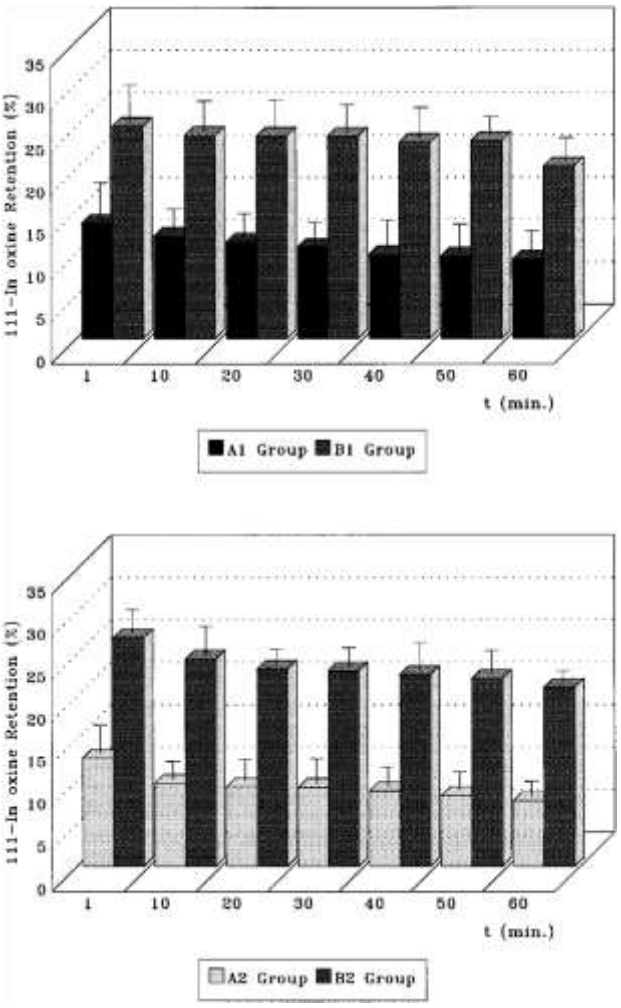

Figure 4. Percentage of radionuclide retention. This percentage refers to the number of cpm (counts per minute, y axis) at a given time (t) in relation to the initial time (0, before circuit, x axis): (a) EC seeded on coated and uncoated ePTFE (groups A_1 and B_1) after exposure to blood flow. Statistically significant differences were observed between both groups ($p < 0.001$). (b) MC seeded on coated and uncoated ePTFE matrix (groups A_2 and B_2) after exposure to blood flow. Statistically significant differences were observed in both groups ($p < 0.001$).

76.90% (± 8.24%) for EC seeded in direct contact with the ePTFE grafts (group A_1) to 71.65% (± 6.23%) for MC seeded on uncoated prostheses (group A_2). There was no significant difference in the surface area of the prosthesis covered among any of the groups before the onset of blood flow (Table I).

After blood flow

After blood flow a reduction in the surface area covered was observed in all groups. This reduction was significant when the groups with cells seeded in direct contact with the

biomaterial were compared to those where cells had been seeded onto the fibroblastic matrix (groups A_1 vs. B_1 and A_2 vs. B_2) (Table I).

DISCUSSION

One of the most important requisites of a vascular prosthesis is that it should possess an antiadhesive luminal surface. This characteristic, which is highly desirable at the prosthetic surface/blood interface, is, in fact, a major disadvantage for seeding of the luminal surface. Previous studies[17] have shown that EC do not readily adhere to PTFE. Fibronectin facilitates the adhesion of cells to the prosthetic substrate and affects their cytoskeletal organization, enabling these cells to form the characteristic confluent monolayer coating the biomaterial. Despite the use of fibronectin to favor adhesion, the stability of the EC layer is not durable. A natural fibroblastic matrix was developed[13,18] to improve the adhesive properties of fibronectin and to increase cell stability after seeding on the PTFE in a static system. In the present study, fibroblastic matrix coated and uncoated prostheses seeded with MC or EC were tested in a blood-flow system.

The results obtained after introduction of the EC/ MC-seeded control-group prostheses in the model circuit, showed that exposure to blood flow produced a loss of up to 88% of radionuclide activity in the first 10 min. From this moment on, radionuclide loss became more stable. These results are similar to those reported by other authors using different models,[8,19] but they contradict those of some studies where cell retention was reportedly better after exposure to blood flow.[20] The radionuclide activity loss produced in the first 10 min in the coated prostheses was significantly less than that mentioned above (75%).

The effect of blood flow on seeded prostheses has generated much debate. Sentissi et al.[21] and other authors[22] tested seeded prostheses under low-flow and high-flow conditions and found that cell retention was better under conditions of low blood flow. There are authors, however, who report better cell retention under high-flow conditions (200 mL/min).[23,24]

TABLE I
Percentage of Prosthetic Surface Covered Before and After Exposure to Blood Flow (Mean ± SD). Measurements Were Taken in 96 Microscopic Fields (×100) in Each Study Group

Groups	Before Blood Flow	After Blood Flow
A_1	76.90 ± 8.24	28.19 ± 9.36*
A_2	71.65 ± 6.23	34.96 ± 10.10†
B_1	78.23 ± 7.50	56.98 ± 9.84*
B_2	73.50 ± 9.36	61.48 ± 7.25†

A_1 = EC/uncoated prosthesis; A_2 = MC/uncoated prosthesis; B_1 = EC/coated prosthesis; B_2 = MC/coated prosthesis. *Comparison A_1/ B_1, $p < 0.005$; †Comparison A_2/ B_2, $p < 0.005$.

In the present model two types of flow measurements were made, one at inflow to the prosthesis (117.5 ± 12.5 mL/min) and the other at the outflow from the prosthesis (72.6 ± 14.3 mL/min). These measurements, which have not been reported in other models, show the resistance of the prosthesis to blood flow. In this model, which can be considered a highflow model, cell retention was better on prostheses coated with a fibroblastic matrix before seeding.

The determination of percentage of cellular coverage of the prosthetic surface was used to complement the ^{111}In oxine retention measurements. EC and MC adherence was improved in the coated prostheses. We used fibronectin to favor the organization of the cellular cytoskeleton and therefore to ease recuperation of the normal cellular morphology with a monolayer structure over the seeded surface. Other authors have reported improved cell retention *in vitro* when the prosthesis is pretreated with collagen.[21]

Although EC and MC have a strong affinity for ^{111}In oxine, two fundamental factors must be considered: the biomaterial's uptake of the radionuclide and the spontaneous loss of radionuclide by labeled cells.[16] Several authors have investigated the spontaneous loss of radionuclide by EC in blood vessels of different species. Their results range from a spontaneous loss of 5% within 60 min in fresh EC from human veins[11] to a loss of 7.8% within the same time period in EC from canine veins.[25] Vhora et al.[23] demonstrated that in EC from the human saphenous vein, greatest spontaneous radionuclide loss occurred in the first 30 min after onset of blood flow in the model, becoming nonappreciable over the following 4 h. In the present model spontaneous radionuclide loss was negligible.

It may be concluded that the loss of radionuclide activity after exposure of prostheses to blood flow is intimately related to the progressive loss of cells from their surfaces. This investigation also demonstrates that coating the prosthetic surface with a fibroblastic matrix before seeding with EC or MC significantly improves the conservation of radionuclide activity and therefore cell adherence to the prosthetic surface when exposed to blood flow. No significant differences in cell retention were found between prostheses seeded with EC and those seeded with MC.

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