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Use of a fibroblastic matrix improves the results of mesothelial-cell seeding on vascular prostheses of polytetrafluoroethylene

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Summary. Mesothelial cells (MC) obtained from the human omentum are a good alternative to the use of endothelial cells (EC) as a covering for vascular prostheses of polytetrafluoroethylene (PTFE), given the antithrombogenic properties and good behaviour in vitro of mesothelial cells. We studied the behaviour of mesothelial cells seeded on PTFE prostheses with an interposed fibroblastic matrix for seeding.

The mesothelial cells were extracted from 30-40 g fragments of human omentum by enzymatic digestion with collagenase. The cells extracted were seeded onto small disks of PTFE to which a matrix composed of fibroblastic cells had been fixed with 5% glycerol after the fibroblasts reached convergence. Interposition of a fibroblastic matrix fixed with glycerol notably improved the adherence of the seeded mesothelial cells and the stability and durability of the cell layer formed on the prosthetic surface.

The effectiveness of seeding mesothelial cells was confirmed by labelling the cells with ^{111}In-oxine. This showed that once the cell layer had formed (24 h after seeding), the fibroblastic matrix favoured the maintenance of a stable layer of mesothelial cells 4 hours after uptake of the radioactive substance.

Key words: Mesothelial cells, Vascular prostheses, Fibroblastic matrix, Cell seeding

Introduction

The use of mesothelial cells (MC) to cover vascular prostheses is an alternative that may solve the problem of having to obtain autologous endothelial cells (EC) from the venous territory (Clarke et al., 1984; Nicholson et al., 1984; Thomson et al., 1989; Visser et al., 1991; Bellón et al., 1994). On the other hand, mesothelial cells have antithrombogenic properties, such as prostacyclin production (Bull et al., 1988; Van Hinsbergh et al., 1990; Bearn et al., 1993).

In earlier studies of seeding of mesothelial cells form human omentum on vascular prostheses of polytetrafluoroethylene (PTFE), we found that the mesothelial cells adhered well and formed a stable covering on the prosthetic material (Hernando et al., 1994). However, the stability of this covering was only transitory and after a week it began to disanchor and lose cells. We have observed similar behaviour after seeding endothelial cells and fibroblasts from humans and from other animal species (Buján et al., 1992; Bellón et al., 1993a,b).

In an attempt to improve the stability of prosthetic coverings, we tested a fibroblastic matrix as a support for seeding endothelial cells (Bellón et al., 1993c), and found that it notably increased the stability of the endothelial monolayer.

In the current study, we proposed to study the behaviour of mesothelial cells seeded on prosthesis of PTFE previously covered with a fibroblast matrix.

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Materials and methods

Cell cultures

Mesothelial cells

For the mesothelial cell cultures, 30-40 g fragments of omentum were removed from different donors during abdominal surgery. The harvested fragments were submerged in minimum essential medium (MEM, Gibco Brl.) supplemented with an antibiotic/antimycotic preparation: 10000 IU/ml penicillin, 10000 µg/ml streptomycin, 25 µg/ml amphotericin B, and Fungizone (Gibco Brl.) and sent to the laboratory.

The omental fragments were washed several times in MEM and the cells were extracted by CLS type I collagenase digestion (0.1% in 15 mM $CaCl_2$ Worthington) for 20 minutes in a 37 ºC shaker bath. Following digestion, the cell suspension was introduced into a 50-ml conical centrifuge tube (Falcon) and

centrifuged for 7 minutes at 200 g. The supernatant was eliminated and the pellet was resuspended in 5 ml of medium 199 (M-199) supplemented with 20% bovine foetal serum, 10 mM Hepes (Gibco Brl.), 10000 IU/ml penicillin, 10000 µg/ml streptomycin (Gibcon Brl.), 2 mM L-glutamine (Gibco Brl.), and 90 µg/ml sodium heparin (Roche).

The cell suspension was placed in a 25 cm^2 culture flask (Nunclon) and incubated at 37 ºC in a 5% CO_2 atmosphere.

Fibroblasts

Fibroblast cultures were started from a small piece of skin (1 cm x 0.5 cm) removed from a donor patient during surgery. This skin was introduced immediately into a container with MEM. The skin was then cut into small fragments to form explants which subsequently were placed in culture flasks containing complete McCoy's medium (Gibco Brl.) and incubated at 37 ºC in a 5% CO_2 atmosphere. The culture medium was changed every 2 days until a confluent monolayer had formed on the walls of the culture flask.

Once the monolayer had formed in the culture flask, the cells were detached with a trypsin/EDTA IX solution (Gibco Brl.), which was allowed to act for 5 minutes at 37 ºC. The action of the enzyme was stopped by addition of complete medium and the resulting cell suspension was separated into culture flasks at a ratio of 1:2.

The number of viable cells obtained from the omental fragments was determined by means of trypan blue exclusion and counting in a Neubauer chamber.

Cell identification

Cell identification was carried out by means of an immunohistochemical technique using anti-CD34, anti-Factor VIII, anti-vimentin, anti-desmin, anti-α actin, and anti-total actin (the last two specific for muscle cells).

CD34. The routine avidin-biotin complex immunohistochemical technique was used to detect CD34 surface antigens. As a primary antibody, the anti-CD34 monoclonal antibody QBEND 10 (Serotec) was used; the secondary antibody was biotin-conjugated rat IgG (Sigma) and napthol esterase (Sigma) was used as chromogenic substrate.

Factor VIII. The routine peroxidase/antiperoxidase complex immunohistochemical technique was used to detect Factor VIII surface antigens following the method used by Baughman et al. (1984).

Vimentin. An anti-vimentin antibody (BioMEDA) was used as primary antibody, gold-conjugated rat IgG (Zymed Laboratories, Inc.) was used as secondary antibody, and a silver solution (Zymed Laboratories, Inc.) as chromogenic substrate (Hernando et al., 1994).

Desmin. The primary antibody, anti-desmin, was extracted in rabbit using desmin purified from chicken gizzard (a gift from Dr. Gabianni). Detection was realized by immunofluorescence using FITC-conjugated anti-rabbit goat IgG (Nordic) as secondary antibody in a 1:10 PBS solution.

α-actin. The primary antibody used was the anti-SM-1 isotype, IgG_{2a} kappa (a gift from Dr. Gabianni). A routine immunofluorescence technique was employed using FITC-conjugated anti-rabbit goat IgG (Nordic) as secondary antibody in a 1:20 PBS solution.

Total actin. The primary antibody was extracted from rabbit using the N-terminal peptide from smooth muscle α-actin as antigen (a gift from Dr. Gabianni). The fraction used as primary antibody was the IgG fraction obtained by ammonium sulphate precipitation. As secondary antibody, FITC-conjugated anti-rabbit goat IgG (Nordic) was used in a 1:10 PBS solution.

Postacyclin production

The prostacyclin levels were detected by radio-immunoassay (RIA), measuring the levels of 6-keto-prostaglandin $F_{1\alpha}$ (Amersham), a stable intermediary in the metabolic pathway of prostacyclin synthesis.

Seeding chambers

Small cylindrical chambers, 0.5 cm internal diameter, were used for the construction of disks of PTFE (W.L. Gore and Associates, Flagstaff, Ariz.), which yielded vascular prostheses of 5-mm internal diameter with an internodal distance of 30 µm.

Cell seeding onto PTFE

Fibroblastic matrix

The fibroblasts obtained from the first subculture were removed for seeding and were centrifuged (200 g) after treatment with trypsin and inactivation in complete McCoy's medium. The cell pellet obtained was resuspended in complete medium for cellular strains, thus yielding cells for counting and for seeding.

Each PTFE disk was incubated with fibronectin solution (20 µg/ml) for 1 h. The preincubated prosthetic surface was seeded with a 0.1 ml cell suspension containing 1 x 10^5 cells. Then the prostheses were incubated (37 ºC in a 5% CO_2 atmosphere) until 24 h after seeding.

Once a monolayer had formed (24 h after seeding), it was fixed to the prosthetic surface by treating it with one of the following:

a) a graded ethanol series (70%, 90%, 100%) for 1 min
b) 10% formaldehyde for 1 h
c) 3% glutaraldehyde for 1 h
d) 5% glycerol for 5 min

Following fixation, the surface was washed repeatedly to ensure complete elimination of the fixative components.

Seeding of mesothelial cells

The mesothelial cells obtained from the first subculture were centrifuged at 200 g for 7 minutes, resuspended in M-199, and seeded onto the fibroblast matrix-coated PTFE prostheses ($2x10^5$ cells/well), which had been incubated previously with fibronectin (20 µg/ml) at 37 ºC for 1 hour.

Cell labelling with ^{111}In oxinate

When the mesothelial cells had formed a monolayer on the prosthetic surface (24 h after seeding), they were labelled with ^{111}In oxine (1 mCi/ml) following the technique described by Sharefkin et al. (1983). To each was added 100 µl of radioactive drug (100 µCi/ml), then the plates were incubated for 15 minutes at room temperature, followed by removal of the excess radioactive drug by washing 3 times with PBS.

Scanning electron microscopy

The specimens obtained were fixed in 3% glutaraldehyde for 2 hours and passed to Milloning's buffer (pH 7.3). They were then dehydrated in a graded acetone series, brought to critical point in a CO_2 Polaron E-3000, metallized in palladium gold, and examined under a Zeiss 950 DSM scanning electron microscope.

Experimental design

Two study groups were established: Control group: PTFE prostheses covered with a fixed fibroblastic matrix (n=4); Group I: PTFE prosthesis covered with a fibroblastic matrix seeded with mesothelial cells (n=8).

The time intervals established for studying the behaviour of the seeded cells were: 24 hours, 48 hours, 4 days, 7 days, 10 days, and 15 days. Studies were made by scanning electron microscopy.

Cells were labelled with ^{111}In-oxine 24 hours after seeding. Isotopic studies were made using a gamma-particle scintillation counter at 2 hours, 4 hours, 6 hours, and 22 hours. Specimens were washed two times with PBS after each study interval.

The statistical design included paired data for each study interval and group and independent data for comparisons between groups. ANOVA and the Newman-Keuls test of multiple comparisons were used.

Results

Culture, identification, and prostacyclin production

Mesothelial cells behaved well in culture and formed a confluent monolayer a few days after initiating the culture. This monolayer consisted of polygonal cells with rounded nuclei that were surrounded by a copious glycocalix (Fig. 1). The mesothelial cells reacted positively to all antibodies used, manifesting positivity to specific antibodies for intermediate filaments, such as vimentin (Fig. 2a), and to antibodies intrinsic to muscle cells, for example, α-actin (Fig. 2b).

The levels of 6-ketoprostaglandin $F_{1\alpha}$ detected were superior to 5 ng/ml.

Fibroblastic matrix

The fibroblasts seeded on the PTFE prostheses formed a stable monolayer on the prosthetic surface within 18 to 24 hours of seeding. When the fibroblast matrix was fixed in graded series of ethanol concentrations, the apical surface of the monolayer became wrinkled, although the structure of the cell layer remained stable (Fig. 3a). However, ethanol changed the permeability of the prosthetic material.

Prosthetic permeability was not altered by fixation with 3% glutaraldehyde, 10% formaldehyde, or 5% glycerol. Glutaraldehyde fixation yielded a markedly wrinkled matrix surface with a large amount of cellular

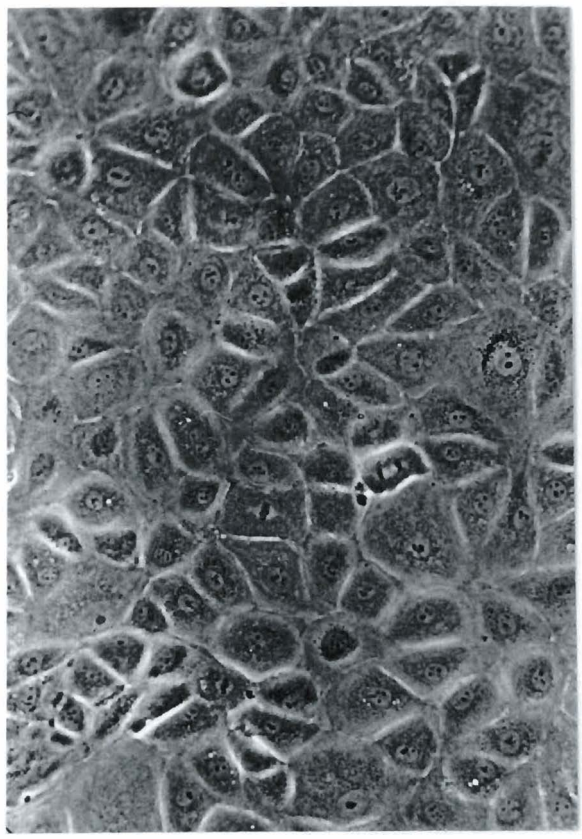


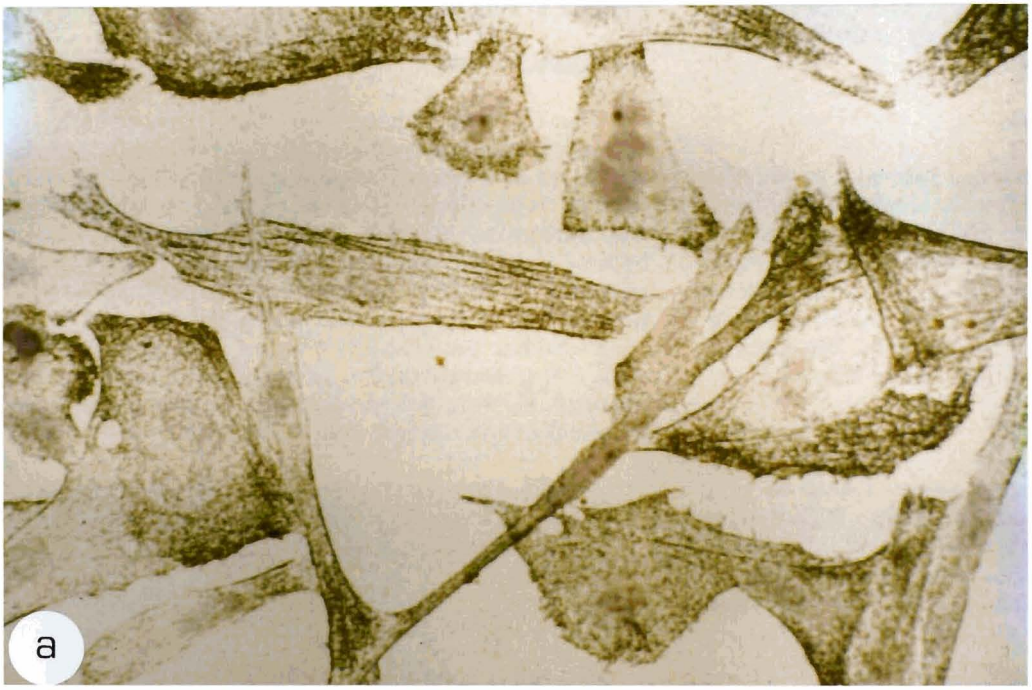
Fig. 1. Mesothelial-cell culture. The cells form a confluent monolayer. This monolayer consists of polygonal cells with rounded nuclei that are surrounded by a copious glycocalyx. x 20

detritus on the exposed surface (Fig. 3b). Formaldehyde fixation produced a matrix that lost part of its stability and showed zones in which the edges of the monolayer became detached (Fig. 3c).

The best results were obtained by glycerol fixation of the fibroblast matrix, which yielded a very stable monolayer that remained intimately joined to the prosthetic surface, with the nodes of PTFE being visible beneath the fixed monolayer (Fig. 3d).

Seeding of mesothelial cells

Twenty-four hours after seeding the mesothelial cells, a very stable cell layer covering the entire prosthetic surface had formed over the fibroblast matrix. The mesothelial layer showed cells of spherical



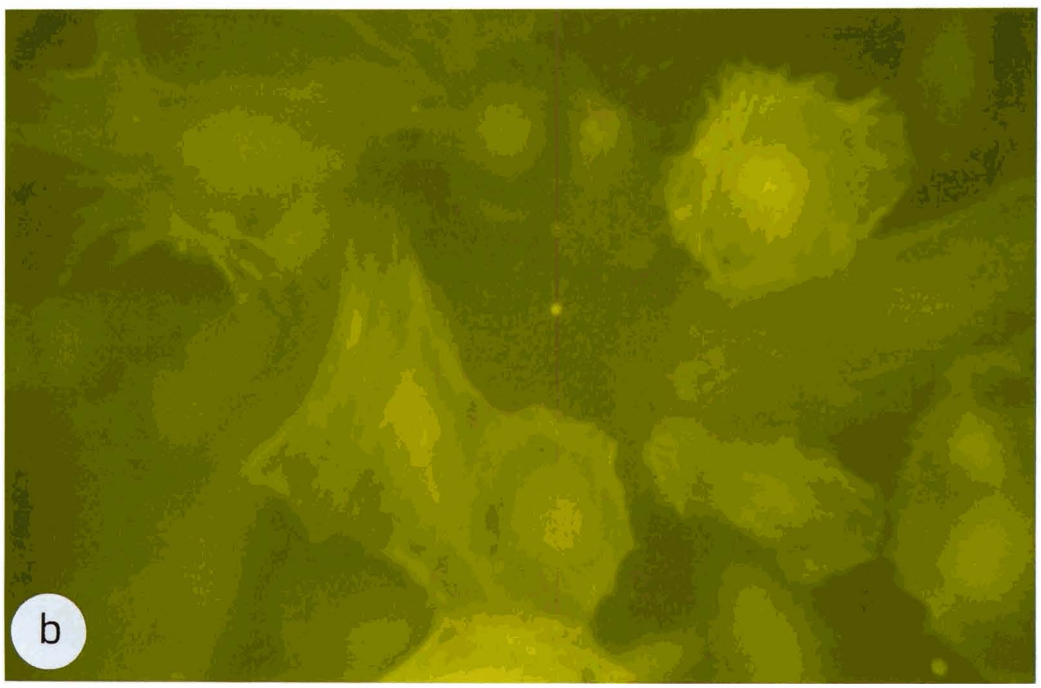


Fig. 2. Immunohistochemical marking of mesothelial cells in culture: **a.** Positive reaction of mesothelial cells to anti-vimentin antibodies. x 40. **b.** Positive immunofluorescent reaction to anti-actin antibodies. x 40

morphology that had the typical «bubbling» appearance of cells at different stages of division, as well as cells that had begun to extend over the surface formed (Fig. 4a). After this stage, the mesothelial cells continued to form a stable covering in either monolayer or strata over the seeded surface. By 15 days, not only were the mesothelial cells well-established on the matrix, but they had good viability and renovation capacity (Fig. 4b).

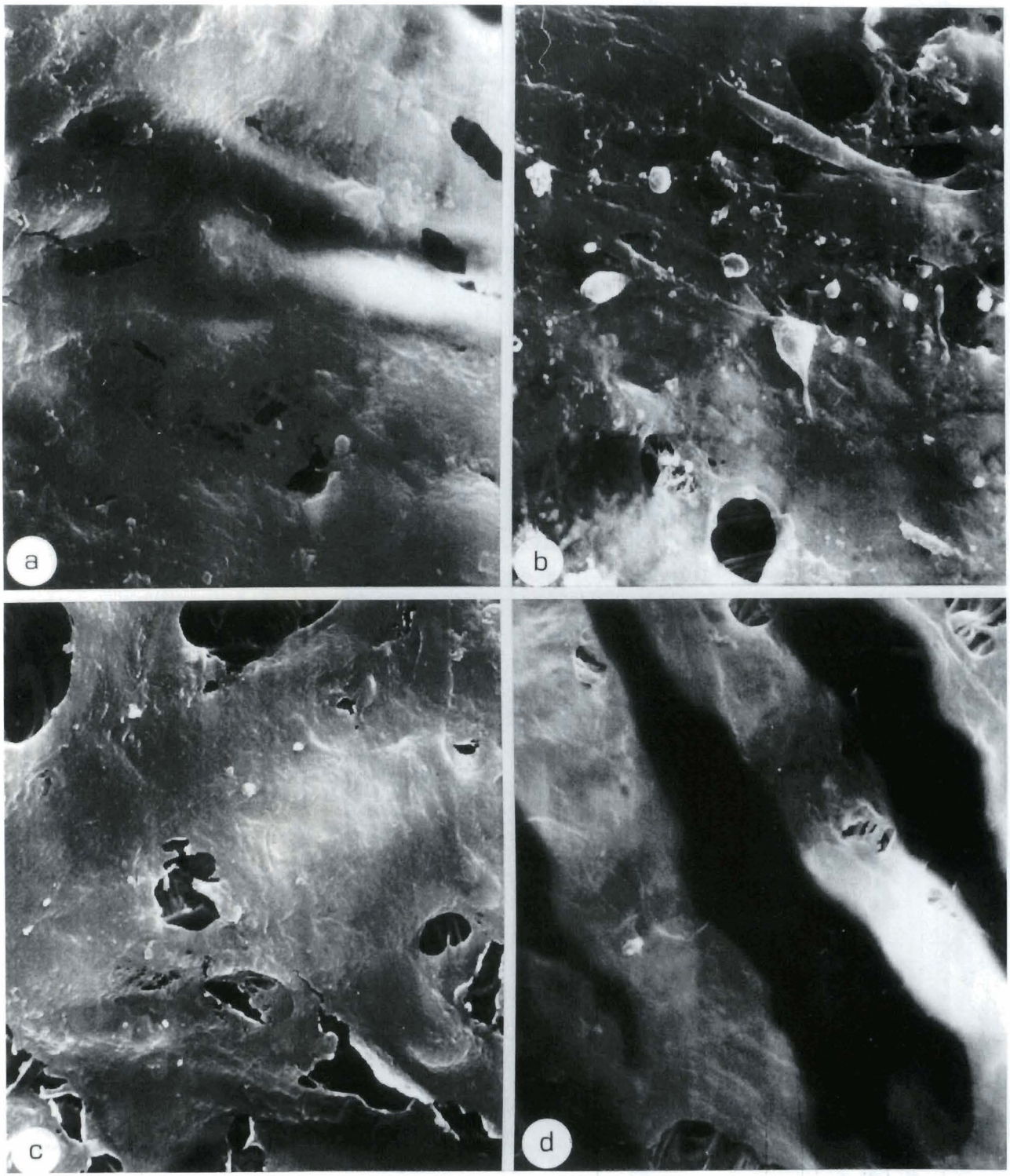


Fig. 3. Monolayer of fibroblasts on PTFE prosthesis material after treatment with different fixers (SEM): **a.** Graded ethanol series. x 1,000. **b.** 3% glutaraldehyde. x 1,000. **c.** 10% formaldehyde. x 1,000. **d.** 5% glycerol. x 1,000

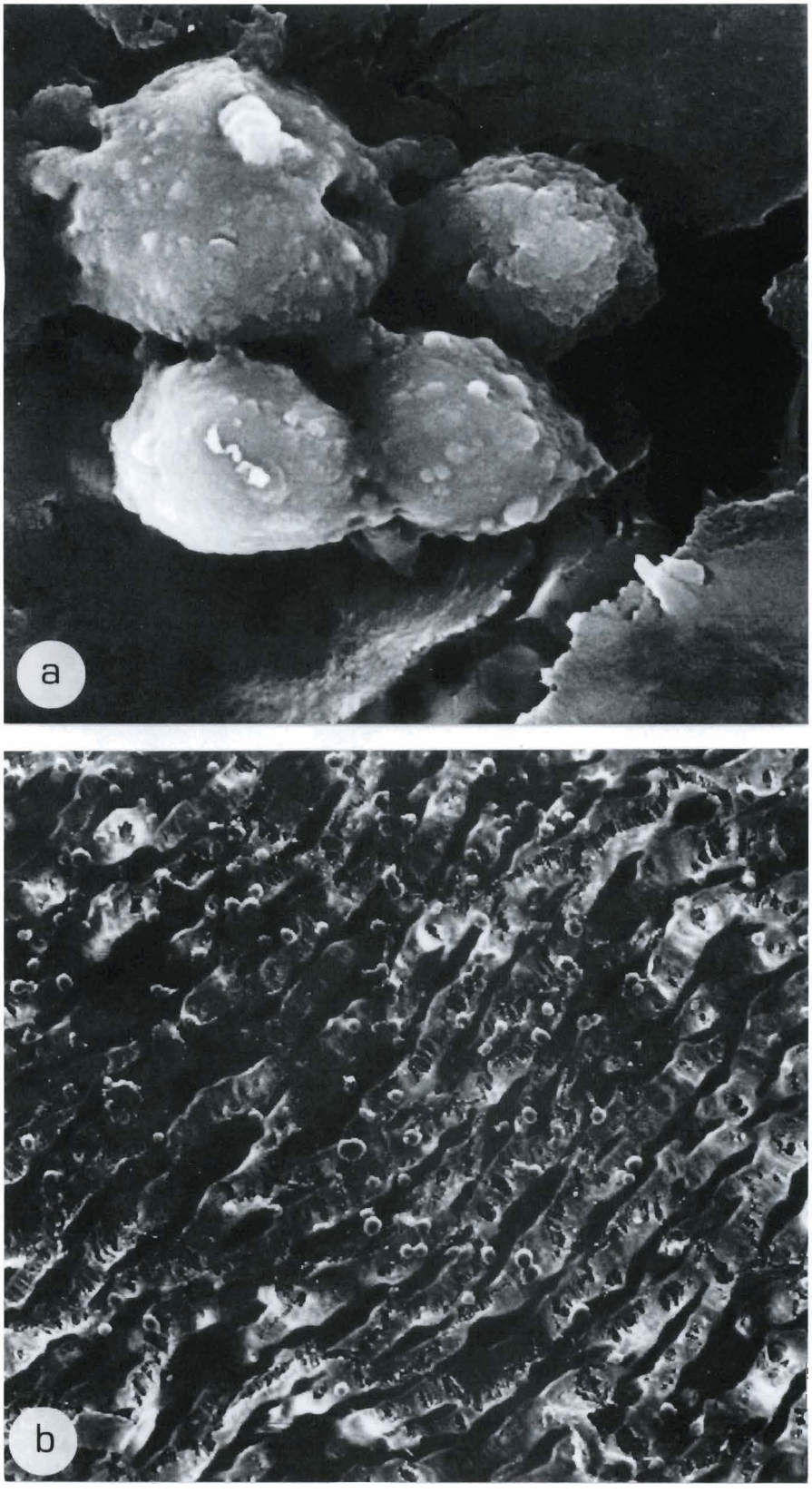


Fig. 4. a. Mesothelial cells of spherical morphology in different stages of division at 24 hours. x 3,000. **b.** Cell layer maintained 15 days after seeding. mesothelial cells have good viability and renovation capacity. x 200

^{111}In oxine uptake

Measurements of ^{111}In oxine uptake showed in the control group that a large amount of radioactivity was retained by the fibroblastic matrix after incubation with the isotope. The initial uptake decreased considerably at 2 hours, but from then on the loss of radioactivity stabilized and the radioactive substance persisted on the fibroblastic matrix up to 22 hours. During this period, labelling decreased more, but not to the point at which there were significant differences in relation to the loss of radioactivity at 2 hours after incubation with the radioactive substance (Fig. 5).

The mesothelial cells (Group I) showed a similar behaviour of uptake in time. After incubation, there was a strong uptake of ^{111}In by the mesothelial monolayer. However, uptake decreased considerably from 2 hours on, then stabilized until 22 hours; no significant differences being observed between 4 hours, 6 hours, and 22 hours (Fig. 5).

For all the study times, radioactive uptake by the fibroblastic matrix was significantly superior ($p<0.05$) to that detected in the mesothelial cells (Fig. 5) when Group I was compared to the control group.

Discussion

Previous studies have shown that endothelial function is better when the subendothelium is similar to the natural vascular middle layer (Baker et al., 1985; Weinberg and Bell, 1986; Jauregui, 1987). This was confirmed by our experiments, in which interposition of a layer of fibroblasts as a natural matrix between the vascular prosthesis and the endothelial cells (Bellón et al., 1993c) was found to provide a substrate that facilitated good adherence, favoured by the action of fibronectin, and better long-term stability.

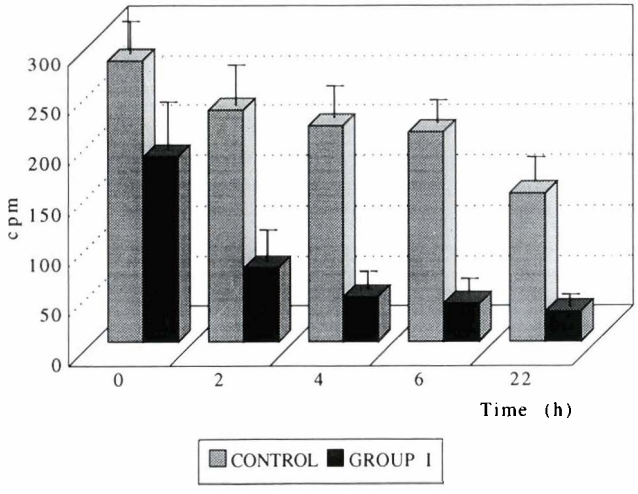


Fig. 5. Amount of In^{111}oxine retained by the mesothelial cells in relation to the amount of radioactive uptake by the fibroblastic matrix in time. cpm: count per minute ($x10^{-3}$).

The use of a living cell layer as preliminary substrate for seeding mesothelial cells would be optimal for seeding vascular prosthesis. However, fibroblasts, like other cell lines used, do not remain stable on the prosthesis surface for more than a few days (Bujan et al., 1992). Therefore, the fibroblast matrix has to be fixed to guarantee its immobility. Ethanol, used as a fixer in earlier experiments (Bellón et al., 1993c), had the disadvantage of altering prosthetic permeability (Van der Lei et al., 1991), which complicates later seeding methods because the culture medium filters through prosthesis interstices. This experience led us to try to improve the fibroblast matrix by using other fixers, such as glutaraldehyde, formaldehyde, or glycerol. Glycerol was chosen because it produced minimal changes in both the fibroblast monolayer and the prosthetic structure.

Other authors have used natural matrices for seeding. However, their studies have not focused on the use of a cell layer to cover the prosthetic surface before final seeding, but on the use of the matrix secreted by seeded cells (Vlodavsky et al., 1987; Schneider et al., 1992; Solomon, 1992). In the same line of research, but using a cell line that is closer to that of our model, Lee et al. (1993) created a matrix by destroying fibroblasts previously seeded on another biomaterial (polyurethane).

The results we obtained from seeding mesothelial cells did not differ from those obtained with endothelial cells (Bellón et al., 1993c) in terms of establishing and maintaining a cell covering throughout the study period. However, mesothelial cells, in contrast to endothelial cells, can be arrayed in various strata when seeded.

Superficial spherical cells with good viability and functional activity were found to be frequent. This indicates that the conditions provided by the underlying substrate were adequate for maintaining these cells. Thomson et al. (1989) made a similar observation, but they did not use a fixation matrix and their studies were very short (1 hour). The same authors described the presence of spherical cells as a disadvantage for adherence under flow conditions. However, we interpreted the finding of spherical cells as perhaps characteristic of the early stages, as was demonstrated by the isotopic studies. Our long-term findings using a fibroblast matrix are that these spherical cells are anchored firmly to the underlying substrate which allows them to endure flow conditions.

As regards ^{111}In-oxine uptake, the protein surface of the fibroblast matrix and fibronectin showed avid uptake of the radioactive marker. Uptake decreased in the first hours and remained practically stable for the first 2-6 hours. The reduction in radioactive uptake detected at 22 hours can be attributed to the decrease in the radioactive substance's activity (Bearn et al., 1993) and to degradation of some proteins; for example, fibronectin. In earlier studies on the behaviour of fibronectin (García-Honduvilla et al., 1995), we found that fibronectin labelled with I-125 had a similar pattern of activity. We can attribute this to the behaviour of the radioactive substance used.

Isotopic uptake by the mesothelial cells was greater than that of endothelial cells (Budd et al., 1989), so it can be considered a useful marker for studying the evolution on these cells on vascular prostheses.

We conclude that: a) Interposition of a fibroblast matrix created by fixing a fibroblast monolayer onto the prosthesis with glycerol improved the long-term stability of the mesothelial cells seeded on PTFE prostheses. b) Labelling the seeded mesothelial cells with ^{111}In-oxine showed that this layer achieved stability 4 hours after labelling.

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