Seeding of expanded polytetrafluoroethylene (ePTFE) vascular grafts. A morphological study of porcine endothelial and fibroblast cells

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Summary. The need to improve clinical results with small and medium calibre grafts has led to extensive research on cell seeding of prosthetic materials. Numerous problems remain regarding identification, seeding, adhesion and survival of the cells attached.

We have studied the behaviour of seedings of endothelial and fibroblast cells on ePTFE grafts.

Scanning electron microscopy allows us to observe the morphological characteristics and their interaction with the biopolymers. It has been possible to differentiate both cellular types by their characteristics and interactions with the ePTFE. At the same time, from this «in vitro» study it can be concluded that the time needed to obtain a stable and confluent monolayer on ePTFE pretreated with fibronectin is between 18 hours to 4 days for endothelial cells, and 24 hours for fibroblasts. These would be the optimal time periods for «in vivo» grafting of the seeded prostheses.

Key words: Endothelial cells, Vascular prostheses, Fibroblast cells, Cell seeding

Introduction

Endothelial cell seeding of prosthetic vascular grafts is a technique that tries to improve the results of vascular reconstructive procedures using medium and small-calibre prostheses (Herring et al., 1978). Its aim is to create a cellular interphase between blood and the prosthetic material.

Endothelial cell seeding of vascular grafts in laboratory animals seem to be effective (Graham et al., 1980; Stanley et al., 1982; Allen et al., 1984; Köveker et al., 1991). Nonetheless, it has not been possible to achieve the same results on clinical assays.

There are several aspects that require a study in depth. Among these, the origin and number of endothelial cells necessary to obtain a stable cellular

lining for an effective seeding, as well as the substrates with which seeding must be performed.

It is not always easy to obtain an adequate number of autologous cells for fresh seeding (Herring et al., 1980; Sharefkin et al., 1983). So, «in vitro» culture techniques are used on the cells obtained, as a step previous to seeding (Maruyama, 1963; Jaffe et al., 1973; Lewis et al., 1973).

Other sources, besides veins, for the obtainment of endothelial cells can be microvessels from adipose or pericardial tissue (Thomson et al., 1989; Sugimoto et al., 1989; Visser et al., 1991). In such cases, it is very difficult to obtain highly purified cultures of endothelial cells, as these become contaminated with other cell types from those tissues.

Another important problem is the poor adherence to prosthetic materials, which does not allow the formation of a cellular lining after seeding. This has led to the use of adhesive substances (Thomson et al., 1991) and of natural substrates (Lalka et al., 1989).

It was precisely the search for a natural substrate which led us to do the present work. Our aims have been: first, to study the behaviour of porcine endothelial cells seeded over an expanded polytetrafluorethylene (ePTFE) vascular prosthesis; second, to assess whether a previous seeding of fibroblasts over the ePTFE graft could be used as a natural substrate for an endothelial cell seeding.

For reasons of availability at our Department, mini-pig was the animal used.

Only qualitative aspects such as adhesion and cellular stability over the prosthetic material, irrespective of any quantitative parameters have been studied.

Materials and methods

Endothelial cells

Minipigs, weighing between 20 and 30 Kg, were submitted to general anaesthesia with sodium thiopental (2 mg/kg) (ABBOTT LABORATORIES), endotracheal intubation and inhalation of a mixture of oxygen and nitrous oxide, with i.v. sodium thiopental, pancuronium

bromide (ORGANON S.A.) and phentanilum chloride (SYNTEX LATINO S.A.) on maintenance doses.

A bilateral vertical cervicotomy was performed, carefully dissecting both external jugular veins, ligating collaterals and cannulating their ends, where 3-way-stopcocks were applied.

The veins were placed in a container with MEM (GIBCO By Life Technologies LTD), supplemented with 5 mg/ml Fungizona (SQUIBB E.R. and SONS), and 100 UI/ml penicillin and 10 µg/ml streptomycin (GIBCO).

The vein segment was the repeatedly flushed at the laboratory, in sterile conditions, with MEM (GIBCO), to remove red blood cells; it was then filled to distention with a 0.1% collagenase II solution (15 mM $CaCl_2$) (SIGMA CHEMICAL CO) and incubated at 37° C for 15 minutes.

Once the contents were evacuated into a 10 ml sterile test tube, the vein was flushed several times with serum-free medium M199 (GIBCO). The cell suspension was done at 200 g for 7 minutes, and supernatants were discarded. The cell pellet was resuspended in complete medium M 199 (CMS): M 199 standard (GIBCO); 90 µg/ml heparin - porcine intestinal mucosa (SIGMA); 2 mM L-Glutamine (GIBCO); 20 µg/ml endothelial growth factor (SIGMA); 20 nM Heppes Buffer (GIBCO) and 100 UI/ml Penicillin - 10 µg/ml streptomycine (GIBCO). A small aliquot was taken for cell counting, and cell suspension remainders were seeded into 25 cm^2 culture flasks (NUCLON - Intermed). The medium was changed every other day until the cells were virtually confluent.

Fibroblast cells

A small piece of skin (1 x 0.5 cm) was removed from the cervicotomy incision during surgery. This skin was immediately introduced into a container with MEM (GIBCO) and then cut into small fragments that formed explants. These were subsequently put into culture flasks containing MCCOY'S MEDIUM (GIBCO), and the medium was changed every other day.

Cell identification

Since porcine endothelial cells do not express factor VIII-related antigen on their surfaces «in vitro» (Pedersen and Fass, 1980), no attempt was made to stain these cells with an immunofluorescenst Factor VIII-related antigen. However, Factor VIII-RA PAP (Factor VIII-Related Antigen Peroxidase anti-Peroxidase) (Baughman et al. 1984) was used for rapid cell identification in the cross-sections of jugular veins obtained before and after collagenase digestion.

The fibroblasts were positive to the vimentin immunohistochemical reaction (Biomeda), that was negative in the endothelial culture.

Cell counting

Cell counting was done with freshly harvested cells just before seeding.

A solution containing 20 µl of the suspension and in 180 µ blue-trypan at 0.2% was prepared, and an aliquot was place into a NEUBAUER (WEBER SCIENTIFIC INTERNAT. Ltc counting chamber.

Subculture cells

The cells (endothelial and fibroblast) were resuspended b treatment with 1.5 ml of 0.05% trypsin/0.53mM EDT, solution (GIBCO), incubated for 5 minutes at 37° C subsequently inactivated with 6 ml of CMS, and the tot; volume was then divided into two 50 cm^2 culture flak (NUNCLON Intermed) for subculture cells.

Seeding chambers

Small cylindrical chambers, 2 cm in length by 1 cr internal diameter (Budd et al., 1989) were used in th construction of a disc of ePTFE (W.L. Gore and Associate: Flagstaff Ariz.), obtained from vascular prostheses of 5 mr internal diameter, with an internodal distance of 30 µm.

Cell seeding

The cells obtained from the first subculture were remove for seeding, and were centrifuged (200 g) after being trypsin treated and inactivated in complete medium. The cell pelle was resuspended in HBSS (GIBCO). This was centrifuge again, and the pellet obtained resuspended in Medium 19 (CMS) for both cellular strains, thus obtaining cells fo counting and seeding.

Each ePTFE disc was seeded with a 0.1 ml cell suspensio containing 5 x 10^5 endothelial cells or 480 x 10^3 fibroblasts.

Experimental design

Two seeding groups were designed:
   A-1: Endothelial cell seeding on ePTFE treated witl fibronectin (20 µg/ml) for 15 minutes prior ti seeding.
   A-2: Fibroblast seeding on ePTFE treated as above.
Study time periods were as follows: 10 min, 1h, 2h, 18h 24h, 48h, 4 days, 7 days and 10 days.

The samples obtained from the two groups were fixed i glutaraldehyde (3%) for 18 hours and passed to MILLONINC buffer (pH 7.3); they were then dehydrated in a graded aceton series reaching a critical point in a CO_2 POLARON E-300 metallized in palladium gold and examined through a ZEIS: 950 DSM scanning electron microscope.

Results

«In vitro» culturing of porcine cells yielded most favourabl results both for endothelial cells and fibroblasts, showin; fusiform morphology, and a peculiar capacity for growing i strata.

On the contrary, the initial growth of fibroblasts from ski explants was slower. They grew in rosettes on culture flask and their morphology could be either polygonal or fusiform
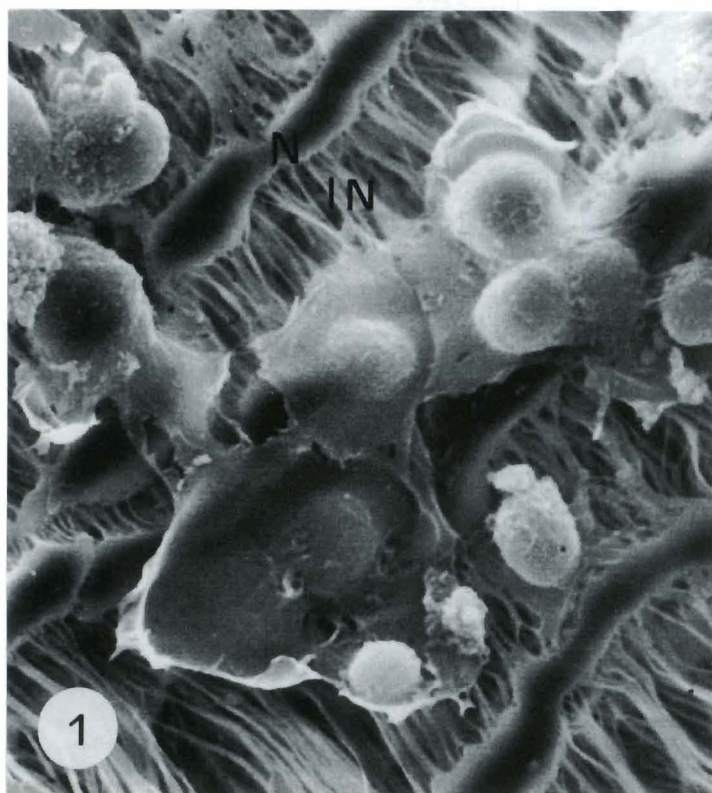
Fig. 1. Endothelial cells. Adherence of endothelial cells to ePTFE nodes (N) 10 minutes after seeding. Internodes (IN). x 1,000
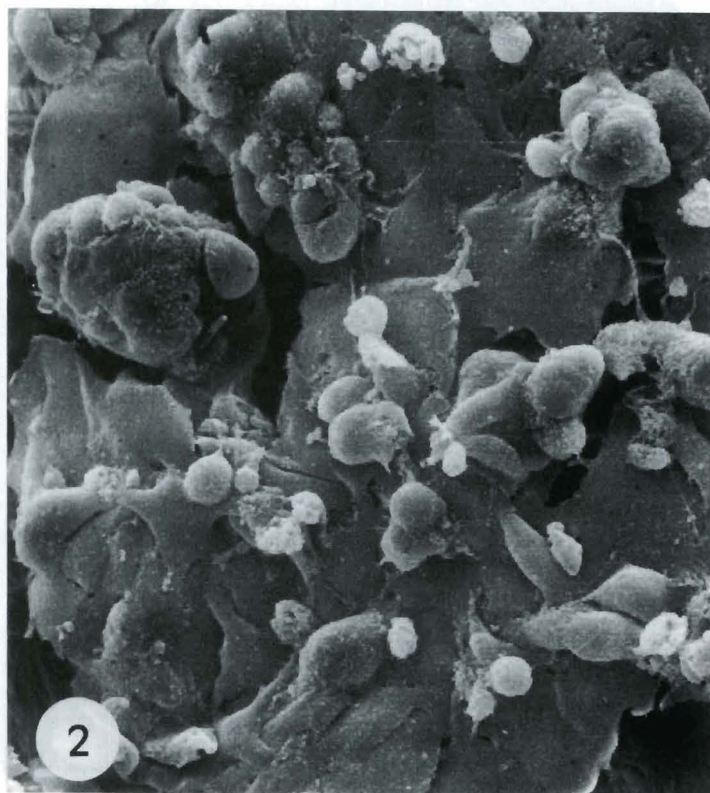

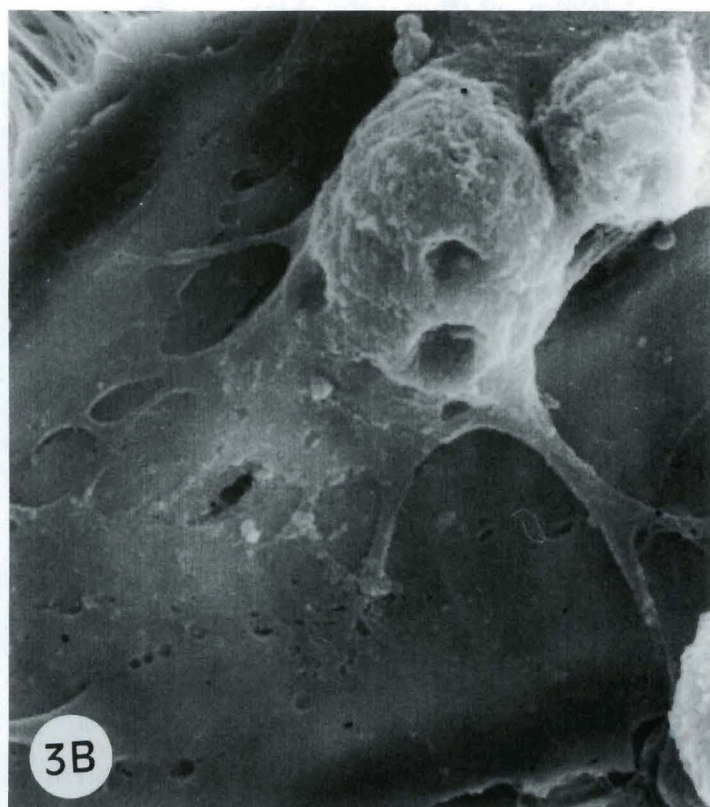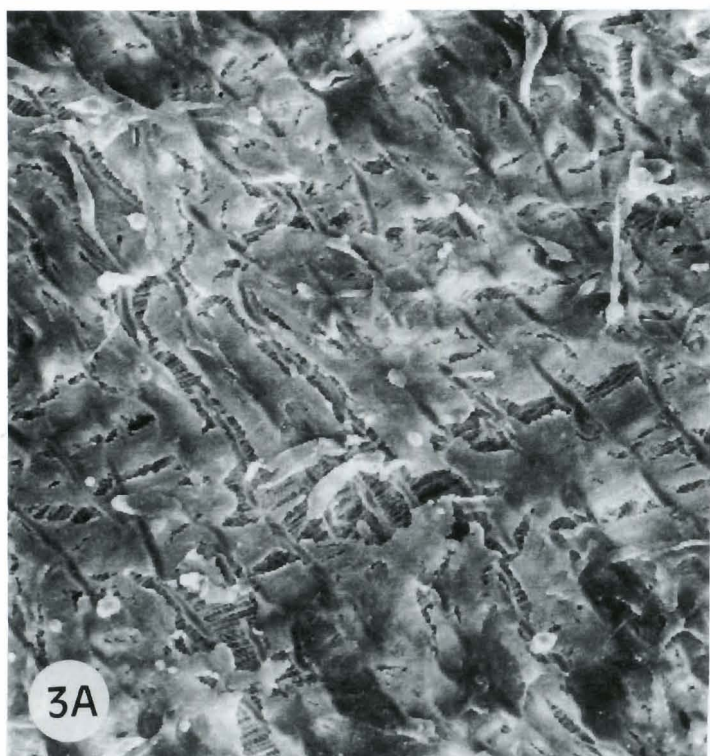**Fig. 2.** Endothelial cells. Growth in multilayer two hours after seeding. x 500

Fig. 3. Endothelial cells. **a)** Cellular confluent lamina obtained 18 hours after seeding. x 200. **b)** Endothelial cell adhering to a cellular stratrum. x 2,000
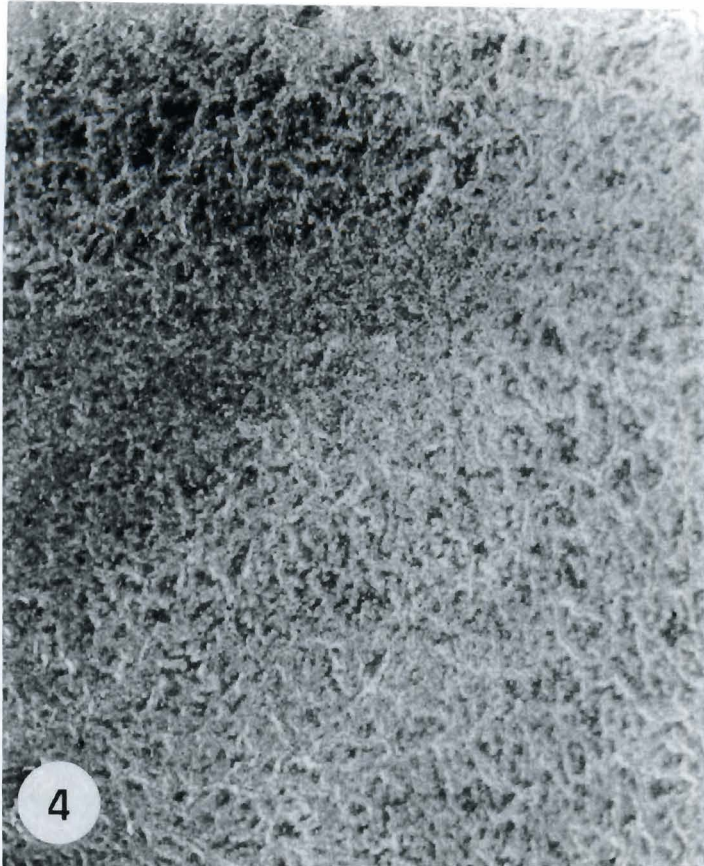
Fig. 4. Endothelial cells. ePTFE disc 24 hours after seeding, monolayer without visible borders. x 16
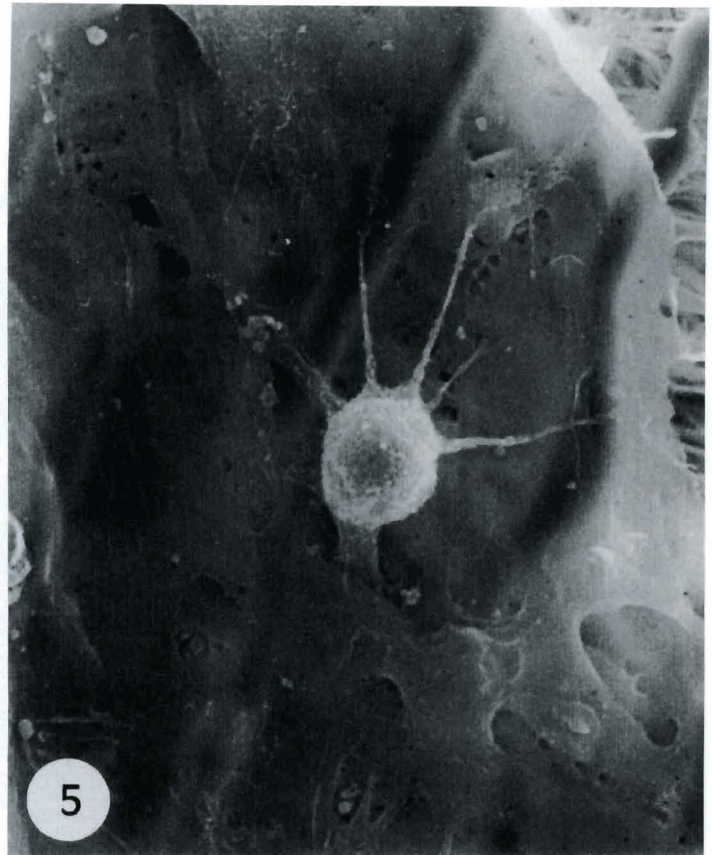

Fig. 5. Endothelial cells. Endothelial cell about to divide, over underlying monolayer after 4 days. x 1,000
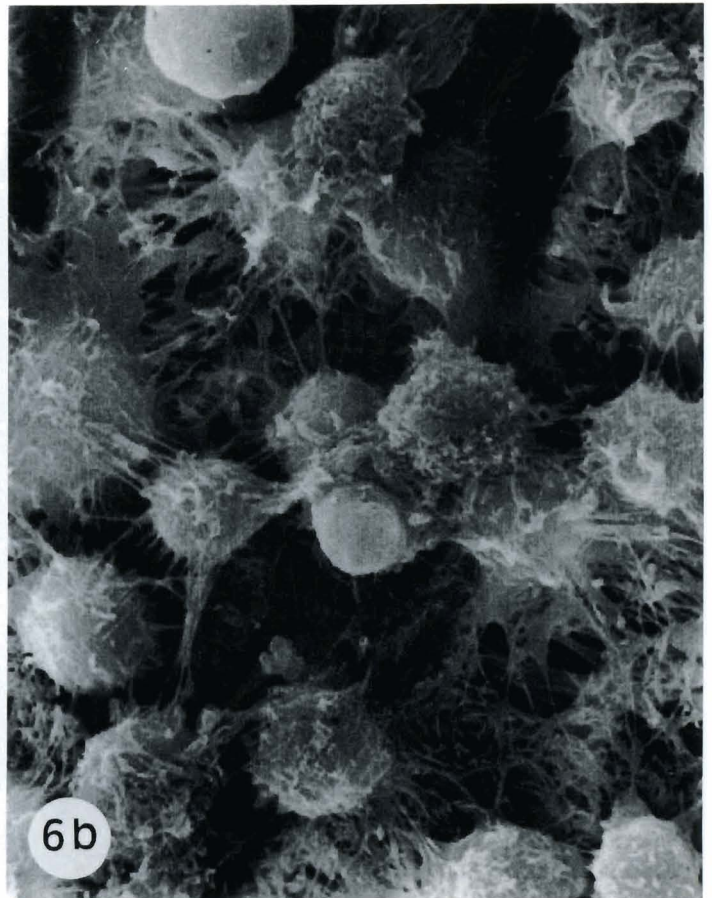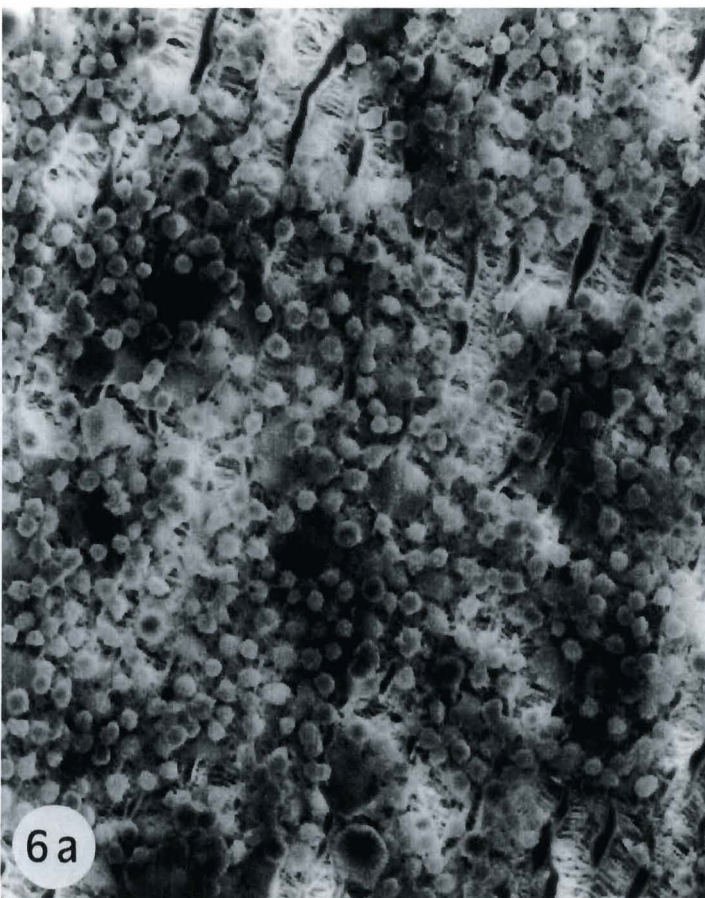
Fig. 6. Fibroblasts. a) Peculiar aspect of fibroblasts 1 hour after seeding. x 200. b) The complex fibrillary network among fibroblasts. x 1,200
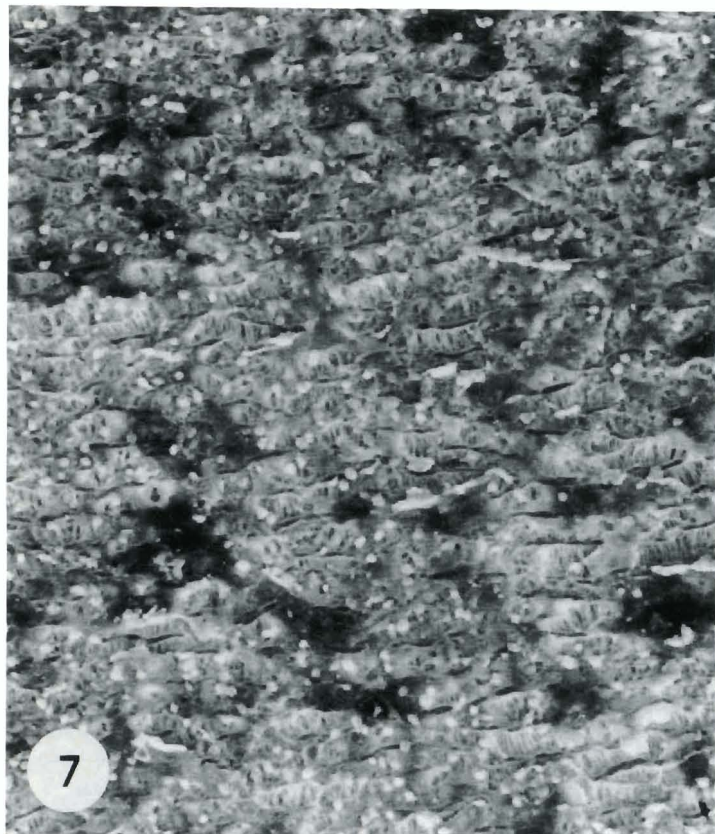
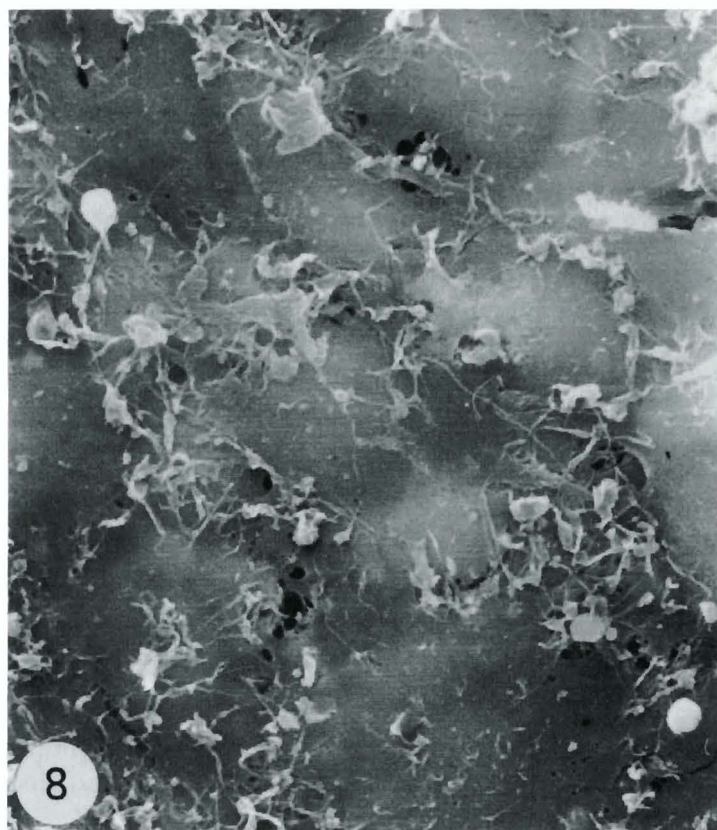**Fig. 7.** Fibroblast. Confluent monolayer 18 hours after seeding. x 50

Fig. 8. Fibroblasts. Fibrillary aspect of the fibroblasts apical surface on monolayer, 24 hours after seeding. x 1,000
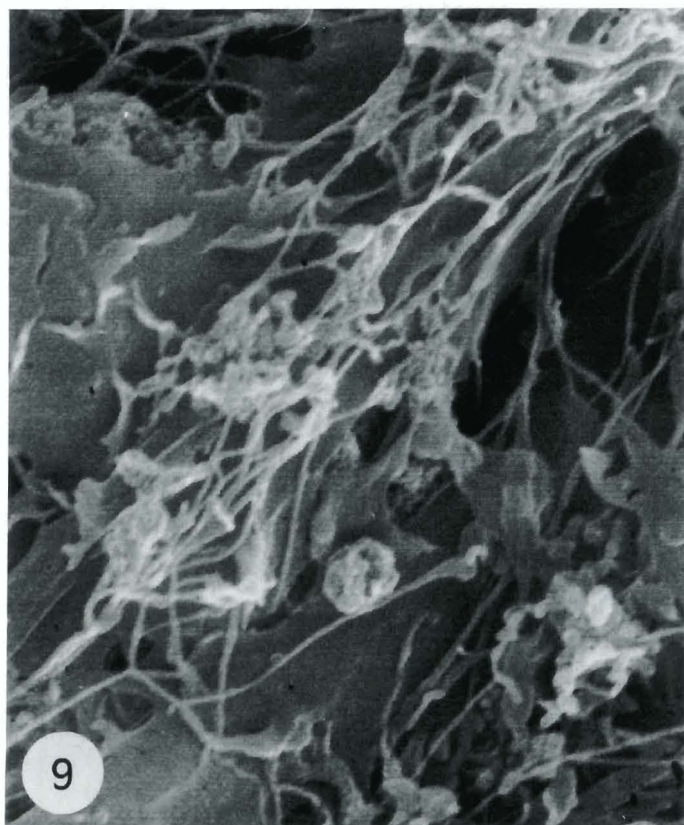

Fig. 9. Fibroblast. The fibrillary component secreted by fibroblasts, 48 hours after seeding. x 5,000

with large nuclei and fibrillar prolongations resulting in reticular-shaped images.

Adherence of endothelial cells to ePTFE

Ten minutes after seeding, a large number of cells could be seen on the ePTFE prostheses. Most of them were not yet attached to the substrate and showed a spherical morphology. However, an initial adherence could be seen in some of the cells through the morphological changes they presented, with polygonal flattened shapes and mainly located on the ePTFE mesh nodes (Fig. 1).

After one hour, the number of attached cells increased. These appeared in different shapes: polygonal; fusiform; and spherical. Some of them showed morphological features of their different cellular cycle, such as the typical bubbles that appear on the cellular surface while in cytokinesis. This adhesion took place not only with the underlying substrate but also between the cells themselves, through large intercytoplasmic bridges.

Two hours after seeding, an organization in layers could be observed, although exposure areas on the ePTFE mesh were still relevant. Over the cellular lamina one could see cells at different functional levels (Fig. 2).

At 18 hours after seeding a confluent monolayer appeared on the ePTFE disc covering all the surface

(Fig. 3a). On some sites, cells in expansion were visible over the underlying endothelial cells (Fig. 3b).

At 24 hours, the coverage presented a clear laminar aspect formed by a cell coating where intercellular junctions were scarcely seen (Fig. 4). This monolayer remained for 4 days. Round cells over the cellular lamina with the features of a cell initiating its division were observed (Fig. 5). At this stage, elevation and leakage of laminar fragments of endothelial cells could be seen, causing denudation of the ePTFE surface. After 7 days the ePTFE disc was only partially covered by cellular laminae remaining in some areas.

Fibroblasts adherence to ePTFE

Results achieved by endothelial cell seeding were similar to those obtained with fibroblasts, although substantial differences can be established in the morphology of the cell, form and time of adhesion, as well as permanence of the substrate.

The first differences could be seen in the initial phases of the seeding process. Fibroblasts tended to locate themselves not only over the ePTFE nodes but also on the fibrillar internodal mesh (Fig. 6a).

The initial round, flat cellular morphology started changing, with the appearance of fine radial prolongations that gave them a spicular aspect (Fig. 6b). These prolongations increased in number and thickness intertwining with those of the adjacent cells and forming a fibrillar network which maintained the fibroblasts attached. These still retained their spherical shape, which was a characteristic feature an hour after seeding.

Subsequently, cells started to develop their own cytoskeleton to adopt a flattened-polygonal morphology that enabled them to adhere to the substrate. At 18 hours after seeding, cellular laminae could be seen on the ePTFE mesh, showing a less flattened surface than that observed with endothelial cells, due to the many fibres on it. At this stage, a great deal of spherical cells could be seen over the flattened laminae, with ePTFE intercalary nodes also visible (Fig. 7).

The confluent monolayer became established in 24 hours, although the cellular limits were indistinct. This monolayer is less stable on the ePTFE disc than that of endothelial cells (Fig. 8).

At 48 hours, the appearance of denuded ePTFE areas and of cells with abundant fibrillary secretion (Fig. 9) could be seen. After this, a loss of cells took place and, by the fourth day, groups of retractile cells appeared over the ePTFE disc (Figs. 10a,b). This represented the first sign of detachment from the prosthetic substrate. At 7 days only small single cells remained over the synthetic
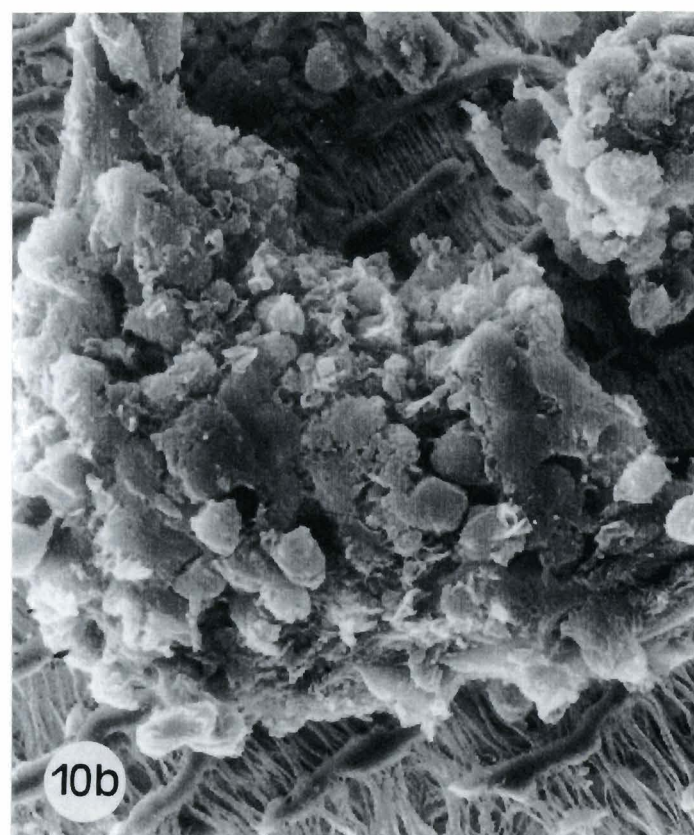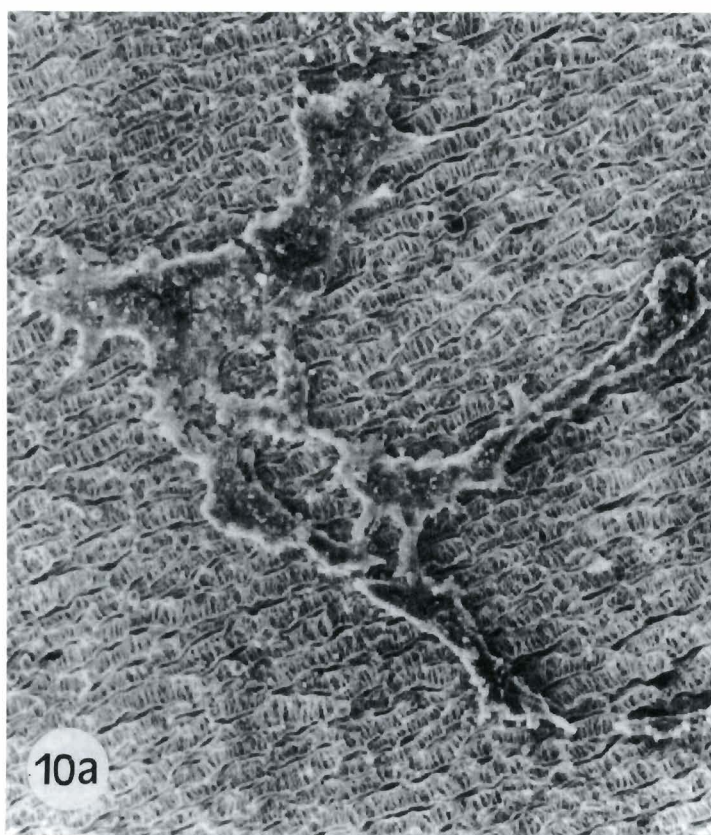

Fig. 10. Fibroblast. **a)** General view showing extensive denuded areas 4 days after seeding. x 100. **b)** Visualization of loss of adhesion and functional activity 4 days after seeding. x 500

material.

Discussion

Shortcomings in obtaining autologous endothelial cells, as well as their low adhesion capacity on biomaterials, have led to the search for new sources of cells and adhesion substrates.

To overcome this problem it is important to be able to differentiate cell populations closely located in the different tissues and organs, such as endothelial cells and fibroblasts.

Although they are easily identified in healthy tissues, the problem becomes more complex, from a morphological point of view, when these two types of cells are studied in culture.

Our results have shown the differences observed in cell cultures. The ability of porcine endothelial cells to grow in strata contrasts with the results achieved by other authors (Jaffe et al., 1973; Lewis et al., 1973; Wachem Van et al., 1986; Budd et al., 1989; Lalka et al., 1989; Sugimoto et al., 1989; Thomson et al., 1989, 1991; Visser et al., 1991), who relate the appearance of multilayers to fibroblasts and smooth muscle cells. This behaviour could be explained on the basis of differences between species. Different degrees of adhesion and permanence, over synthetic substrates, of endothelial cells from different animal species, have been reported (Sauvage et al., 1974; Ortenwall et al., 1988; Kent et al., 1989).

In some cases (Minakawa, 1989) it has been observed that endothelial cells in culture form capillary-like tubular structures. In our studies, we have only seen a great intracellular activity of endothelial cells, with the formation of rosettes of cells surrounding an area of flocculent material. In fibroblast cultures we have observed, from the beginning, a remarkable tendency to grow forming concentric rossetes, although no growth in multilayer was seen.

Besides the morphological aspects, cell identification is completed with different methods to separate cellular strains, such as intravital colorations (Mechem et al., 1989); monoclonal antibodies (Dejana et al., 1990), isotopic markers (Budd et al., 1991) etc. Up to now it remains unclear whether cells being cultured, or those submitted to further subcultures, modify or not the features by which they are identified (Mechem et al., 1989; Minakawa, 1989; Mischeck et al., 1989; Dejana et al., 1990; Budd et al., 1991).

We have used fibronectin as biological adhesive in the seeding of the different cells onto ePTFE. Ramalanjaona et al. (1986), reported the lack of endogenous fibronectin production by endothelial cells when these were removed from their usual environment. The difficulty of adhesion of endothelial cells onto the ePTFE mesh, found by other authors and ourselves, (Foxal et al., 1986; Hernando et al., 1991) has encouraged us to systematically use fibronectin as adhesive, in concentrations that seem to be most favourable (Budd et al., 1990).

When seeding starts, some of the differences existing between the two cell types become evident. Endothelial cells attach to the ePTFE mesh nodal areas, whereas fibroblasts preferably do so at the fibrillary areas. Furthermore, adhesion and cellular proliferation seem to be related to some characteristics of the material such as wettability and charge (Wachem Van et al., 1989).

The change from spherical to round form is faster in the endothelial cell than in fibroblasts. In the latter there is first a phase of fibrillary secretion, before the organization of its cytoskeleton. That gives a peculiar aspect to the seeding, from the beginning to 18 hours afterwards, allowing an easy identification.

Fibroblast adhesion to ePTFE seems rather scarce, and this raises doubts about its likely use as natural substrate previous to endothelial cell seeding (Haylfick, 1965; Watson et al., 1987; Sedlarik et al., 1990).

Most works on quantification of cell attachment to ePTFE have had very short observation periods (Sedlarik et al., 1990; Vohra et al., 1991). Although our methods were similar, we have found that, beyond the fourth day after seeding, this adhesion process is not stable.

In conclusion: a) There are remarkable differences between endothelial and porcine fibroblast cells, in their adhesion to a ePTFE substrate; b) The time needed to obtain a confluent monolayer is 18 hours for endothelial cells and 24 hours for fibroblasts; c) Stability for fibroblasts lasts only 24 hours, remaining 4 days for endothelial cells; d) Based on these findings, we do not believe that a previous seeding with fibroblasts could be used as a natural substrate for endothelial cell seedings.

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