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In vitro mesothelialization of prosthetic materials designed for the repair of abdominal wall defects

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The aim of this study was to evaluate the *in vitro* response of mesothelial cells (MC) in terms of their ability to cover different biomaterials. MC were harvested from human omentum. The MC from the first passage were seeded onto different biomaterials from 10 min to 24 h: PL-PU99 (polypropylene-polyurethane); DM (ePTFE); PL (polypropylene); and PL + Col (polypropylene-collagen). The prosthetic surface covered was examined by microscopy and quantified. *PL-PU99*: The MC were adhered to the biomaterial 10 min post-incubation. At 4 h, the 53.12 ± 7.86% of the prosthesis were coated with polygonal cells. At 12 h, 96.32 ± 11.32% of the biomaterial was coated. *DM*: between 30 min to 8 h, the MC cells form small, round colonies. At 12 h, polygonal and fusiform secretory cells were observed (68.94 ± 5.78%). 93.54 ± 11.49% of surface was coated after 24 h. *PL*: only isolated cells were observed on the prosthesis. *PL + Col*: MC form a monolayer over prosthetic surface after 18 h (90.21 ± 9.76). We conclude: (a) MC formed a stable monolayer over all the biomaterials tested with the exception of the PL due to its porosity. (b) The PL-PU99 showed the greatest potential for *in vitro* mesothelialization compared to the PL-Col and DM prostheses.
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1. Introduction

The repair of large abdominal wall defects due to incisional hernias or to the resection of a tumor, generally involves the use of a prosthetic material. In some cases, the prosthetic material needs to be placed in direct contact with the visceral peritoneum. In this situation, the interface formed between prosthesis and peritoneum may be the site of subsequent complications such as adhesion formation possibly leading to intestinal occlusion [1]. The appearance of intestinal fistulas has also been described [2, 3] and there are even reported cases of migration of the biomaterial used in the repair process to hollow organs [4–6].

In studies performed *in vivo* [7], the interface with the peritoneum shows variable tissue repair behavior and, to a large extent, this behavior depends more upon the structure of the biomaterial employed than on its composition. In order to avoid complications, it is crucial that this interface is appropriately generated.

The aim of the present study was to evaluate the *in vitro* response of the peritoneal mesothelium in covering different types of biomaterials. This capacity is likely to be a significant predictive factor of the *in vivo* behavior of some of these implants, in terms of the correct formation of a neoperitoneum.

2. Materials and methods

2.1. Cell harvesting and culture

Mesothelial cells (MC) were obtained from 30–40 g fragments of human omentum obtained from donors during the course of elective abdominal surgery. These fragments were transported to the laboratory immersed in minimum essential medium (MEM) (Gibco BRL) supplemented with antibiotics (100 000 IU/ml penicillin/10 000 μg/ml streptomycin) (Gibco BRL) and antimycotics (25 μg/ml fungizone) (Gibco BRL). The specimens were then processed following the methods described by Kern *et al.* [8] with some modification.

In the laboratory, the omental fragments were incubated in a 0.1% solution of type I collagenase (Worthington) in MEM, with agitation (100 oscillations/min) for 20 min. Surplus omental fragments were withdrawn and the cell suspension centrifuged at 200 g for 7 min. The supernatant was discarded and the resultant cell precipitate was resuspended in 5 ml of medium 199 (M-199) (Gibco BRL) supplemented with 20% fetal bovine serum (Gibco BRL), antibiotics (100 000 IU/ml penicillin/10 000 μ/ml streptomycin) (Gibco BRL), 10 mM Hepes (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and sodium heparin (90 μg/ml) (Roche). The cell suspension was transferred to Nunclon

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25 cm^2 culture flasks and incubated in a culture oven (5% CO_2) at 37 °C.

The culture medium was changed every 2 days until the formation of a confluent monolayer of cells. Next, sufficient cells to completely cover the prosthetic surface were obtained by treating the MC monolayer with a suspension of 1% trypsin-EDTA (Gibco BRL) for 5 min at 37 °C. After incubation, the proteolytic enzyme was inactivated by adding medium (M-199) supplemented with fetal calf serum (Gibco BRL). All the cells used in this study were obtained from this first subculture.

2.2. Cell identification

The cells obtained by enzymatic digestion and subsequent fractioning in a density gradient were identified using histochemical techniques. Peroxidase-antiperoxidase procedures were used to detect cytokeratin 18 (Dako); and desmin, α-actin and total actin (all courtesy of Professor Gabiani) were determined by immunofluorescence (FITC).

In all the techniques applied, the same biological material without the addition of the primary antibody (replaced with 100 μl PBS) served as the negative control.

2.3. Preparation of the prosthetic surface

Several polypropylene *(PL)* prostheses *(Marlex®, Bard Cardiosurgery Division, Billerica, MA)* and an expanded polytetrafluoroethylene (ePTFE) prosthesis (*Dual-Mesh® WL Gore and Assoc., Flagstaff, AZ*) were used to establish the study groups. Each biomaterial was cut into 24 fragments (2 cm^2) that were placed in multi-well plates for subsequent cell seeding: *PL group*, fragments of a prosthesis formed by a *PL* monofilament of 1 mm pore size; *PL + Col group*, fragments of a *PL* prosthesis treated with equine collagen *(PL-Col)* to prevent the seeded MC passing through the 1 mm pores; *PL + PU group*, fragments of a *PL* composite prosthesis PL-PU99 designed by our team. This prosthesis is composed of a layer of polypropylene and a 26 μm layer of polyurethane joined with an acrylic adhesive; *DM group*, fragments of an ePTFE prosthesis.

Prosthetic surfaces were prepared for seeding by treatment with 100 μl fibronectin solution (20 μg/ml) and incubation in a CO_2 oven for 1 h at 37 °C.

The study was performed at 10 and 30 min, 1, 4, 8, 12, 18 and 24 h. A mean of 3 fragments/time was used.

2.4. Cell seeding

At the moment of seeding, MC were detached from the culture flask by treatment with trypsin/EDTA and seeded onto the pretreated prosthetic fragments. This involved withdrawing the surplus fibronectin solution and pipetting 200 μl of the cell suspension over each prosthetic surface followed by incubation at 37 °C in a CO_2 atmosphere. The samples were kept under these conditions for the different follow-up times established. The number of cells seeded was 2–2.5 × 10^5 cells/well.

2.5. Coverage of the prosthetic surface with MC

At each follow-up time, prosthetic fragments were fixed in 3% gluteraldehyde for 2 h and placed in Millonig buffer (pH 7.3). Next, the samples were dehydrated in a graded acetone series, brought to critical point in a Polaron E-3000 with CO_2, metallized in palladium gold, and examined under a Zeiss 950 DSM scanning electron microscope (SEM). Measurements of the surface covered were made over 16 micrographs (× 100) of each sample observed by SEM. Each sample was divided into four quadrants and four micrographs were taken in each quadrant at random. The micrographs were then subjected to image analysis (Microm) to determine the surface covered by cells in proportion to the total surface area. Results were expressed as percentage coverage.

The Mann-Whitney U-test was used to compare the percentage MC coverage of each biomaterial at each follow-up time.

3. Results

3.1. MC cultures

During the initial stages of culture, the MC showed a rounded morphology. After the first 24 h, MC adhered to the culture surface forming small colonies of polygonal cells. The number of cells forming the colonies increased and these started to contact one another at subsequent follow-up times. From the third to the fourth day of culture, a monolayer had formed over the surface. This layer was comprised of polygonal cells with a rounded, well-centered nucleus showing 1 to 3 nucleoli surrounded by an abundant glycocalyx (Fig. 1).

3.2. MC identification

MC showed positivity towards all the antibodies tested. Most of the cultured MC showed an intense positive reaction towards the anti-cytokeratin 18 antibodies.

The presence of desmin, α-actin and total actin in all the MC was shown by immunofluorescence. Labeling with anti-desmin antibodies conferred an even, granular appearance to the MC cytoplasm. In contrast, α-actin labeling showed a fibrillar distribution, parallel to the longest cell axes. Total actin was homogeneously distributed around the nucleus.

3.3. MC seeding

3.3.1. PL group

The type of prosthesis used in this study group is formed by a polypropylene monofilament woven to form a mesh exposing 1 mm pores, which impede the cells colonizing the prosthetic surface. Thus, 24 h after seeding, only small groups of polygonal cells were observed on some of the prosthetic filaments (Fig. 2(a),(b)).

3.3.2. PL + Col group

Treatment of the polypropylene prostheses with equine collagen provided a substrate for cell seeding (Fig. 2(c)). Thus, during the initial stages of culture, MC started to extend over the collagen surface emitting long protrusions, allowing contact between distant cells. After 18 h

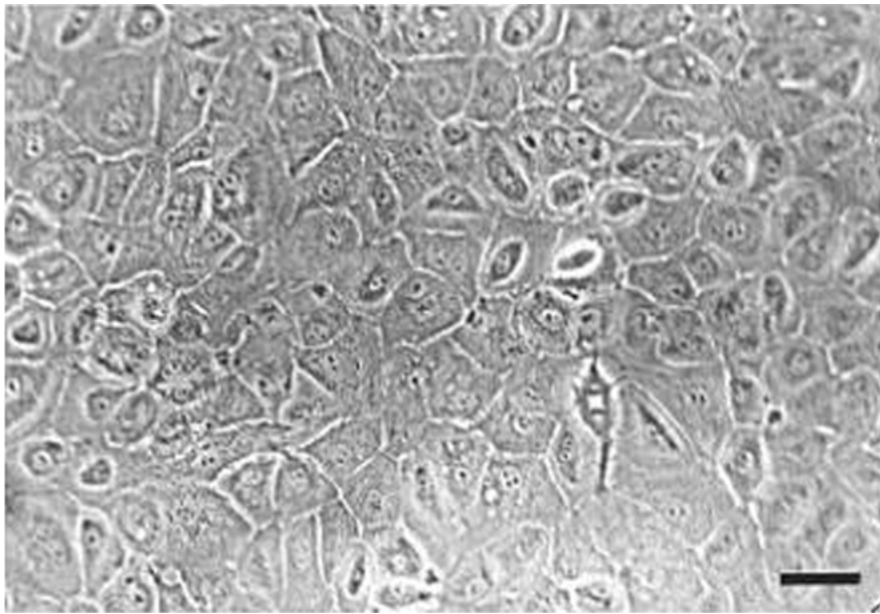
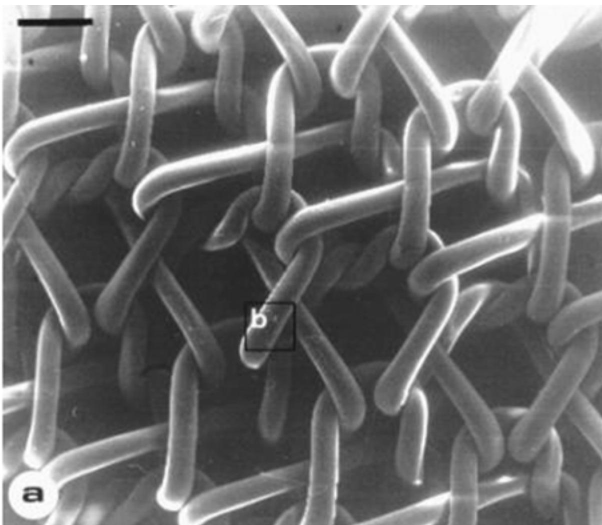
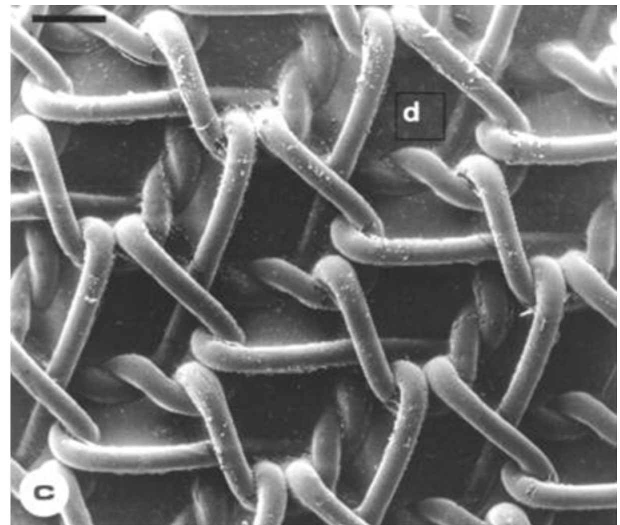
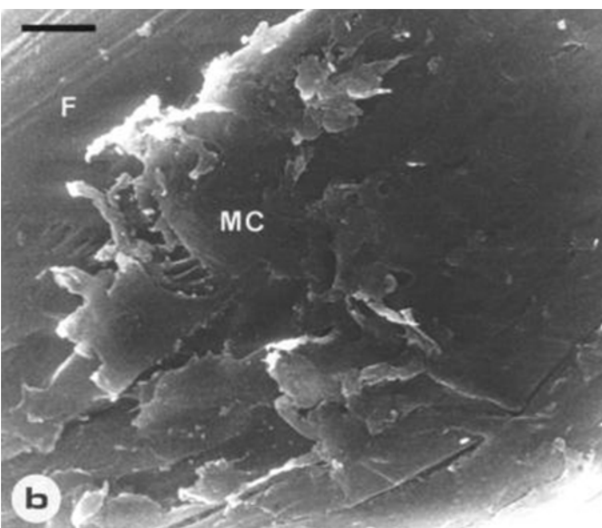


Figure 1 Appearance of mesothelial cells in culture. A monolayer formed by polygonal cells surrounded by abundant glycocalyx (× 10; scale bar: 30 μm).







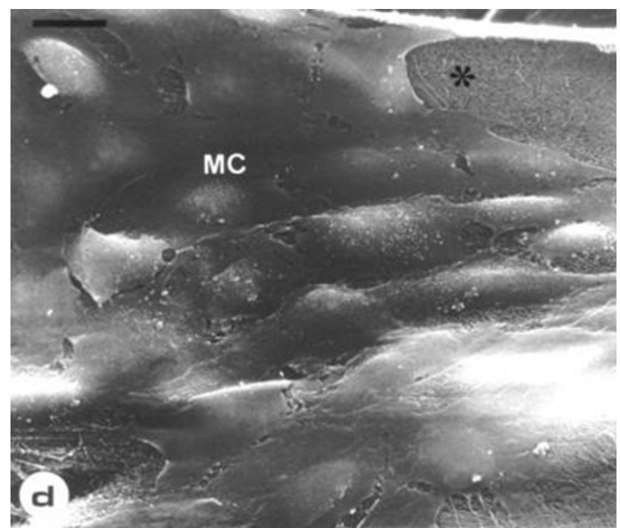


Figure 2 Polypropylene prostheses seeded with mesothelial cells (SEM). (a) and (b) PL group: (a) general view of the polypropylene mesh 24 h after seeding (× 20; scale bar: 500 μm); (b) detailed view of the mesothelial cells (MC) on the prosthetic filaments (F) 24 h after seeding (× 1000; scale bar: 10 μm). (c) and (d) PL + Col group: (c) polypropylene prosthesis coated with collagen, 18 h after seeding (× 20; scale bar: 500 μm); (d) monolayer of mesothelial cells (MC) on the collagen coating (*) 18 h after seeding (× 500; scale bar: 20 μm).

of seeding, the MC formed a monolayer over the collagen coating (Fig. 2(d)). This layer was formed by cells of a highly elongated, polygonal shape.

3.3.3. PL + PU group

In this type of prosthesis, seeding was performed over the polyurethane layer since it is this side that would make contact with the visceral peritoneum when implanted. This layer presents a rough surface on which the MC start to adhere from the initial stages of seeding (10 min). From this time onwards MC start to stretch over the prosthetic surface.

Four hours after seeding, cells already showed the typical MC morphology (Fig. 3(a)) and had colonized wide areas of the prosthetic surface. This progressive colonization of the polyurethane surface reached confluence 12 h after seeding. At subsequent follow up times, almost the entire prosthetic surface was covered by a confluent monolayer of cells showing great secretory activity of their exposed surfaces (Fig. 3(b)). Twenty four hours after seeding, these cells were seen to be interspersed with highly differentiated cells of very smooth surface.

3.3.4. DM group

During the first 4 h of seeding, MC preserved their spherical morphology due to the properties of the Dual-Mesh surface (Fig. 3(c)).

Twelve hours after seeding, extensive zones of the prosthetic surface were observed to be covered with MC, which maintained a fusiform morphology, interspersed with other cells of characteristic polygonal shape. A high degree of secretory activity was noted on the surface of these polygonal MC (Fig. 3(d)).

A confluent monolayer of MC was attained over this type of prosthesis 24 h after seeding. By this time, the entire prosthesis was covered by polygonal cells morphologically consistent with MC, the exposed surfaces of which showed extensive secretory activity.

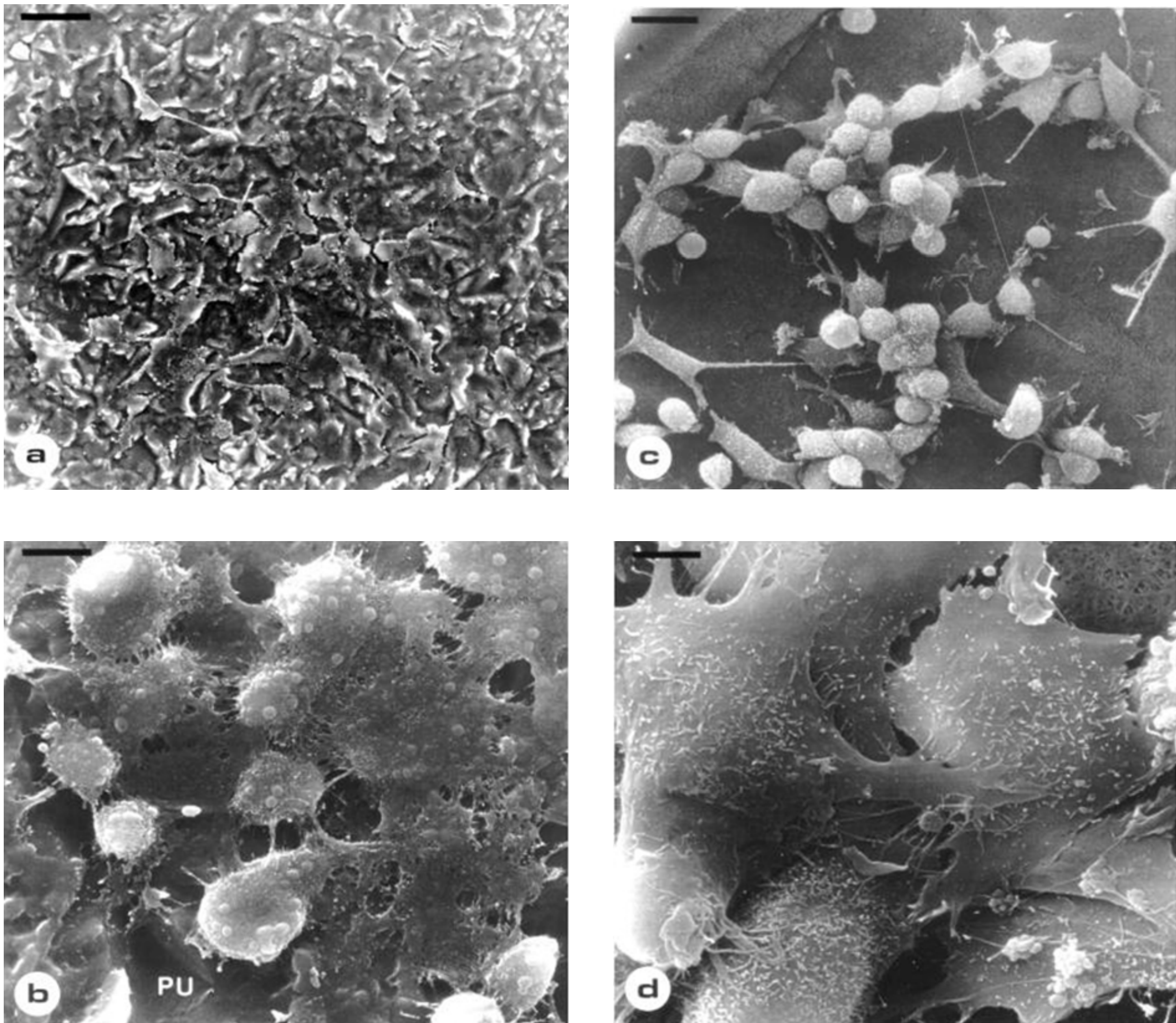


Figure 3 Behavior shown by mesothelial cells (MC) seeded on different biomaterials (SEM): (a) and (b) PL + PU group: (a) prosthetic surface coverage 4 h after seeding (× 260; scale bar: 38.46 μm); (b) appearance of the MC forming a monolayer over the polyurethane (PU) 12 h after seeding (× 1000; scale bar: 10 μm). (c) and (d) DM group: (c) MC during the early stages of seeding (4 h) on the Dual-Mesh surface (× 500; scale bar: 20 μm); (d) detail of the MC monolayer 12 h after seeding (× 2000; scale bar: 5 μm).

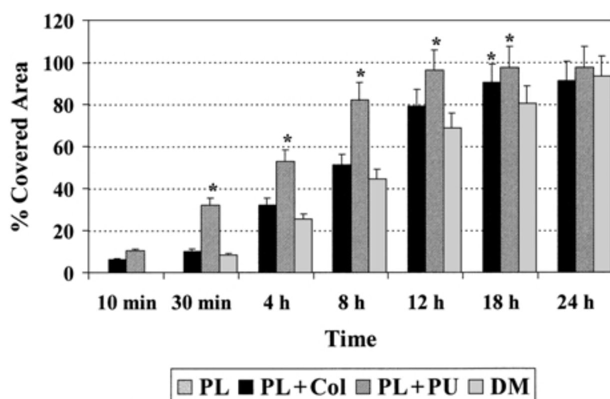


Figure 4 Changes in percentage coverage of the mesothelial cells monolayer on each biomaterial ($^*p < 0.05$).

3.4. Prosthetic coverage

Mesothelial cell coverage of the prosthetic surface determined at the different follow-up times by image analysis indicated large differences among the prosthetic materials employed. The MC showed best behavior over the *composite PL-PU99* prostheses and attained confluence 12 h after seeding and coverage values ranging from 53.12 $\pm$ 7.86% 4 h after seeding to 96.32 $\pm$ 11.32 at 12 h. These values are in sharp contrast with those obtained with the remaining materials. For the DM fragments, coverage was 68.94 $\pm$ 5.78% 12 h after seeding and confluence was not achieved until 24 h (93.54 $\pm$ 11.49%). It was not possible to achieve a confluent monolayer over the *PL* prostheses over the study period, although confluence was reached at 18 h on the collagen-coated *PL* prostheses ($PL + Col$) corresponding to a coverage of 90.21 $\pm$ 9.76% (Fig. 4).

4. Discussion

The ideal prosthesis designed for the repair of abdominal wall defects should achieve optimum integration with host tissue, provide good biomechanical strength and show adequate behavior in terms of minimal adhesion formation when placed in contact with the visceral peritoneum.

In a previous study by our team [9], we demonstrated the rapid formation of a neoperitoneum following the implant of laminar biomaterials in rabbits (48 h and 7 days), but this did not occur when the implanted prosthesis was reticular. We therefore speculated that it is probable that from an early stage, mesothelial deposition after implant is conditioned by the structural design of a prosthesis.

The present study was designed to test this hypothesis and to evaluate the *in vitro* mesothelialization of biomaterials, whether laminar or in the form of a mesh. The model proposed also serves to exclude the effects of other factors, such as cytokines or chemical mediators, possibly implicated in the repair process.

The biomaterials tested were the conventional polypropylene and expanded polytetrafluoroethylene (ePTFE) prostheses commonly used to repair abdominal wall defects. We also examined the mesothelialization process over a *composite* prosthesis formed by a sheet of polypropylene and a polyurethane film.

After seeding the mesothelial cells onto the different biomaterials [9–11], we were able to observe optimal mesothelialization over laminar prostheses of the polyurethane or ePTFE type, as previously noted *in vivo* [12]. This last biomaterial, due to its electronegative charge, was not mesothelialized as rapidly and effectively as the polyurethane, over which a confluent layer had formed by 24 h. In contrast, mesothelialization of the polypropylene mesh was ineffective since it is macroporous; cells escaped through the pores and showed minimum deposition on the biomaterial filaments. This prompted our idea of creating a collagen support in contact with the prosthesis and then seeding on the same side of the mesh but with the pores filled with collagen. Using this set up, the results improved considerably.

In the *in vivo* study performed previously, we were surprised by the speed at which mesothelialization of laminar prostheses occurred. This undoubtedly gives rise to an optimal interface between biomaterial and visceral peritoneum avoiding the formation of adhesions or the appearance of complications related to this problem.

As also mentioned by other authors [13], the importance of the rapid mesothelialization of a biomaterial stems from the fact that adhesion formation is inversely related to the number of mesothelial cells on the peritoneal surface. It is likely that any delay in the formation of a mesothelium when a reticular prosthesis is implanted *in vivo* might be the cause of adhesions at the biomaterial/visceral peritoneum interface. When firm, adhesions may become integrated within the biomaterial and in some cases provoke complications in the long-term including intestinal fistulas [3].

The present findings suggest that: (a) MC were able to form a stable monolayer over all the biomaterials tested with the exception of the PL prosthesis due to its porosity; and (b) the PL-PU99 prosthesis showed the greatest potential for *in vitro* mesothelialization with a monolayer formed 12 h after seeding, compared to 18 h for the PL-Col and 24 h for the DM prostheses.

Acknowledgments

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