Expansion of Human Mesothelial Progenitor Cells in a Long-term Three-dimensional Organotypic Culture of *Processus Vaginalis Peritonei*

(*processus vaginalis peritonei* / mesothelial cells / CD34 / cytokeratins / HBME-1)

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Abstract. A 3D culture system was used to investigate the behaviour of mesothelial cells present in the wall of human *processus vaginalis peritonei.* Small tissue fragments placed on collagen sponges were cultured for 7, 14 and 21 days in medium supplemented with 10% FBS, and analysed for the expression and distribution of cytokeratins (CKAE1-AE3, CK19), p63, Ki-67, vimentin, CD34, and HBME-1. Before culture, flat mesothelial cells displayed immunoreactivity for cytokeratins, vimentin and HBME-1, while p63 and CD34 were negative. Mesenchymal cells within the stroma were vimentin-positive and endothelial cells of small vessels displayed positive staining for CD34. Cytokeratins, p63 and HBME-1 were negative in all stromal cells. In cultured fragments, flat mesothelial cells positive for vimentin, cytokeratins and HBME-1 proliferated, lining the fragment surface and migrating into the sponge. Capillaries showed morphological alterations; however, their immunoreactivity was comparable with the stroma prior to culture. Cells that had migrated into the sponge and displayed characteristics of mesothelial progenitors, predominantly spindle-shaped and stellate, showed heterogeneous expression of markers especially in late phases of cultivation. These cells were constantly positive for vimentin, a small fraction was cytokeratin-positive and a few displayed HBME-1 immunoreactivity. CD34 was found in cells forming small cavities into the matrix, resembling newly formed blood vessels. Cells that had migrated into the sponge could be isolated and expanded in co-culture with feeder NIH.3T3 fibroblasts. This system is suitable for studying growth and behaviour of mesothelial cells within their natural environment, providing a good method for isolation and expansion of their progenitor cells.

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Abbreviations: CK – cytokeratin, mit-c – mitomycin C, PVP – *processus vaginalis peritonei.*

Introduction

Mesothelium is the monolayer of flattened cells with epithelial features lining mammalian serous cavities (pericardial, pleural and peritoneal) and covering the organs within these cavities. In addition, in males it also lines the cavity, *cavum serosum scroti*, that surrounds the testes.

Human mesothelium is derived from the lateral (somatic and splachnic) mesodermal tissue of the embryo (Hesseldahl and Lansen, 1969; Tiedermann, 1976).

Morphologically, flattened epithelial-like mesothelial cells are attached on a thin basement membrane supported by a subserosal connective tissue containing blood vessels and lymphatics, resident inflammatory cells and fibroblast-like cells (Wang, 1974). Mesothelial cells are unique in the sense that they are of mesodermal origin and able to differentiate toward an epithelioid morphology throughout adult life (Tyler et al., 1971; Coulbourne et al., 1992; Bedrossian, 1994). They retain characteristics of both epithelial and mesenchymal cells; however, the surface mesothelium acquires only some of these epithelial characteristics, such as polygonal cell shape and the ability to express cytokeratin intermediate filaments (Walts et al., 1984), desmosomes and a basement membrane (Bolen et al., 1986); but others such E-cadherin expression are less typical or absent (Peralta et al., 1995).

In the foetus and newborn, mesothelium is very important during the development of gonads, genital duct, and in particular it plays a central role in the process of testicular descent. The testes in the male foetus are located in the abdomen. During the last month of pregnancy they migrate in the sac via the vaginal duct (*tunica vaginalis*). The vaginal duct collapses and disappears after several days after the birth (Lawrence et al., 1992; Clarnette et al., 1997), but in particular cases it persists and can cause hydrocele, hernia or funicular cysts.

For over a century mesothelial cells have been used to repair damaged tissues and organs, as well as being among a number of new tissue-engineering applications: such as vascular, omental and peripheral nerve (reviewed in Herric and Mutsaers, 2004). In these last, mesothelial cells seeded onto artificial tubular constructs were used to repair injured nerves. Interestingly, two recent papers reported how cultured mesothelial cells prepared from *tunica vaginalis* and grown on fibrin scaffolds as cell sheets are effective in preventing adhesion formation and reducing postoperative complications in a canine (Asano et al., 2006) and rat model (Takazawa et al., 2005).

In order to study the behaviour, growth and expansion of different cell types present in the wall of human *processus vaginalis peritonei* (PVP), we established a 3-D organotypic culture method that maintains mesothelial cells within their natural microenvironment alive for a prolonged time in culture. This system gave us the possibility to isolate and selectively expand mesothelial progenitor cells *ex vivo.*

Material and Methods

Collection of tissues

Peritoneal-vaginal duct (PVD) samples were obtained from 17 patients (age 0–10 years) who underwent surgery for hernia or hydrocele at the Department of Surgery, Division of Urology, University of Pisa. The parents of the patients provided informed consent. The surgical specimens were cut with blades and scissors into 4–27 mm^3 fragments under sterile conditions. The fragments were washed once with sterile physiologic solution, then with the growth medium and processed following a procedure previously described (Papini et al., 2004).

3D-Organotypic cultures

In order to avoid microbial contamination the tissues were maintained in Dulbecco's modified Eagle's medium-high glucose (DMEM) (Euroclone, West York, UK) supplemented with 500 U/ml penicillin, 0.5 mg/ml streptomycin sulphate, and 1.25 mg/ml amphotericin B (Sigma, St. Louis, MO) for 3–4 h at 4°C before being processed. DMEM was then supplemented with 10% foetal bovine serum (FBS) (Euroclone) ($DMEM_{10}$) and 100 U/ml penicillin, 100 µg/ml streptomycin sulphate, 0.29 mg /ml glutamine. Only one lot of DMEM (Lot. EU S004249, Euroclone) was used in all these experiments. Alternatively, one half of the same sample was incubated in EpiLife medium (Cascade Biologics, Portland, OR) supplemented with EpiLife-Defined Growth Supplement (EDGF, Cascade Biologics), a combination of soluble factors with epithelial growth-promoting activity, including purified bovine serum albumin, purified bovine transferrin, hydrocortisone, recombinant human insulin-like growth factor type-1, prostaglandin E2, recombinant human epidermal growth factor, 10% FBS ($EpiLife_{10}$) and with the same concentration of antibiotics, antimycotics and glutamine. Sterile Gelfoam® sponges obtained from purified pork skin gelatin (Pharmacia & Upjohn, Kalamazoo, MI) were moisturized with $DMEM_{10}$ or $EpiLife_{10}$ medium. Each sponge was cut into three pieces, and each piece was transferred to a well of a 6-well tissue culture plate (Falcon Plastics, Inc., London Ontario, Canada). Prior to culture, the samples were cut along the major axis in order to obtain two parallel samples to cultivate in the two different media. The fragments were then transferred (3/well) and placed on the sponge at the air-liquid interface. The plates were maintained in a humidified incubator at 37°C and 5% CO_2 in air atmosphere. The culture medium was changed every two days. Fragments and sponges were collected at the beginning of the culture (T0), after 7 (T1), 14 (T2), and 21 (T3) days. The samples were fixed in Gliofixx (Italscientifica S.p.A, Genova, Italy) for 24 h at room temperature, and then maintained in 70 % ethanol at 4°C until they were processed for histology and immunohistochemistry.

Isolation and clonal expansion of mesothelial progenitor cells in a two-dimensional monolayer in co-culture with NIH.3T3 fibroblasts as a source of feeder layer

PVD fragments were first cultured on top of the sponges for 21 days in $DMEM_{10}$ or $EpiLife_{10}$. Fragments and sponges were removed from the plate, washed with physiologic solution and digested separately with a trypsin-EDTA solution 1X (Sigma-Aldrich, NY, cat. No. T3924) at 37°C for 80 min. Cells released in suspension were collected every 20 min and the enzymatic reaction was neutralized with $Epilife_{10}$ or $DMEM_{10}$. Cells were plated on a cell culture insert (Transwell-COL, 0.4 mm pore size) in 6-well tissue culture plates, containing a monolayer of post-mitotic, mitomycin C (mit-c)-treated NIH.3T3 fibroblasts seeded in culture 24 h earlier as a feeder cell layer on the bottom of each well, and maintained in a humidified incubator at 37°C and 5% CO_2 in air atmosphere. The medium was changed every second day. At sub-confluence (about 70% saturation of the well) the cells of a single well were detached, transferred to a glass culture chamber slide (Falcon Plastics Inc.; cat. No. 354104), and processed for histological and immunohistochemical analysis. Individual epithelial clones were selected, based on their high growth capacity and phenotypic traits as revealed by immunocytochemical staining (see below), their cells dispersed, then pooled together and seeded in co-culture with the mit-c-treated NIH.3T3 fibroblast feeder cell layer for further expansion.

Histology, histochemistry and immunohistochemistry

3D-Organotypic Cultures

After fixation, samples were dehydrated in 75%, 80%, 96% and 100% ethanol, treated with noxyl (Italscientifica), and embedded in paraffin wax (Histoplast, Shandon, London, UK). The tissues were cut in 5 μm slices and processed with haematoxylin (Harris)-eosin (type Y) for histological evaluation.

Immunoperoxidase staining was performed according to the manufacturer's instructions for each monoclonal antibody. Table 1 shows the list of antibodies used and their specificity. Antigen detection by these antibodies was performed with EnVision™/HRP detection kit (Dako Cytomation, Glostrup, DK) and sections were counterstained with haematoxylin.

Two-dimensional co-culture in chamber slides

Cells at sub-confluence on chamber glass slides (see above) were fixed in ethanol 100% for 6 min at room temperature, washed with phosphate-buffered saline (pH 7.4) and processed for immunohistochemical staining. The total number of immunstained cells was counted at 400× magnification in five unconnected fields; average positive number was calculated for each marker.

Results

Histology of 3D-organotypic cultures

Cultures maintained in $DMEM_{10}$ or $EpiLife_{10}$ yielded comparable results at any given time in long-term culture. The results of haematoxylin and eosin staining are shown in Fig. 1A, 1B and 1C. Prior to the culture (T0), tissue samples were formed of collagen connective tissue containing numerous small blood vessels. A mixture of small areas of mature adipose tissue, skeletal muscle fibres and individual short segments of peripheral nerves was also observed in several samples (Fig. 1A). In three cases, prominent chronic inflammatory infiltrate consisting predominantly of small lymphoid and plasma cells was observed within the stroma. The surface of tissue samples was partially covered by one layer of flat cells corresponding to mesothelial lining of *processus vaginalis*. Fissural infoldings into the stroma lined with identical cells were also exceptionally observed (Fig. 1A).

At T1, individual mesothelial cells were lost in some points (Fig. 1B); however, mesothelial cells on the surface of cultured fragments survived and showed the tendency to migrate around and cover the entire surface of the cultured fragment. The stromal component of cultured tissue samples did not show any significant changes; only the endothelial cells of some blood vessels were altered and detached into the lumen. A small fraction of phenotypically primitive spindle-shaped or stellate cells appeared within the sponge in the vicinity of cultured tissue fragments.

Identical microscopic picture within the cultured tissue samples was also observed at T2 and T3 *in vitro* (Fig. 1C, T2 and T3). Contrary to T1, the number of spindle-shaped and stellate cells migrating from the cultured fragments into the gelatin sponge was much higher at these time points (Fig. 1C insert).

Immunohistochemistry

Cytokeratins (CKAE1-AE3, CK19)

Flat mesothelial cells on the surface of tissue samples and the cells lining the infoldings into the stroma displayed positive immuno-staining for cytokeratins in both non-cultured (T0, Fig. 1D and 1G) and cultured (T1-3, Fig. 1E, 1F, 1H and 1I) tissue samples. Moreover, the majority of spindle-shaped and stellate cells migrating from cultured tissue fragments into the sponge gave positive immunostaining for both antibodies (Fig. 1F and 1I). Stromal elements and blood vessels were negative for these markers (Fig. 1D-I).

Ki-76

Prior to culture (Fig. 1J-L) the expression of Ki-67 was confined to a few cells (<0.5%) and the same proportion of positive cells remained constant without variation throughout culture.

HBME-1

HBME-1, a marker of mesothelial cells, showed a similar immunoreactivity pattern as cytokeratins in both non-cultured and cultured tissue samples (Fig. 2A-2C), with the exception of spindle-shaped and stellate

Table 1. List of antibodies used in this study

Monoclonal antibody	Specificity	Dilution	Source
AE1/AE3	epithelial and mesothelial	1:100	Dako-Cytomation
CK19	epithelial and mesothelial	1:75	Dako-Cytomation
HBME-1	mesothelial	1:50	Dako-Cytomation
vimentin	mesenchymal cells	1:100	Dako-Cytomation
CD34	endothelial cells	1:50	Dako-Cytomation
p63	epithelial and stromal cells	1:50	Santa Cruz Biotecnology
Ki-67	proliferating cells	1:50	Dako-Cytomation

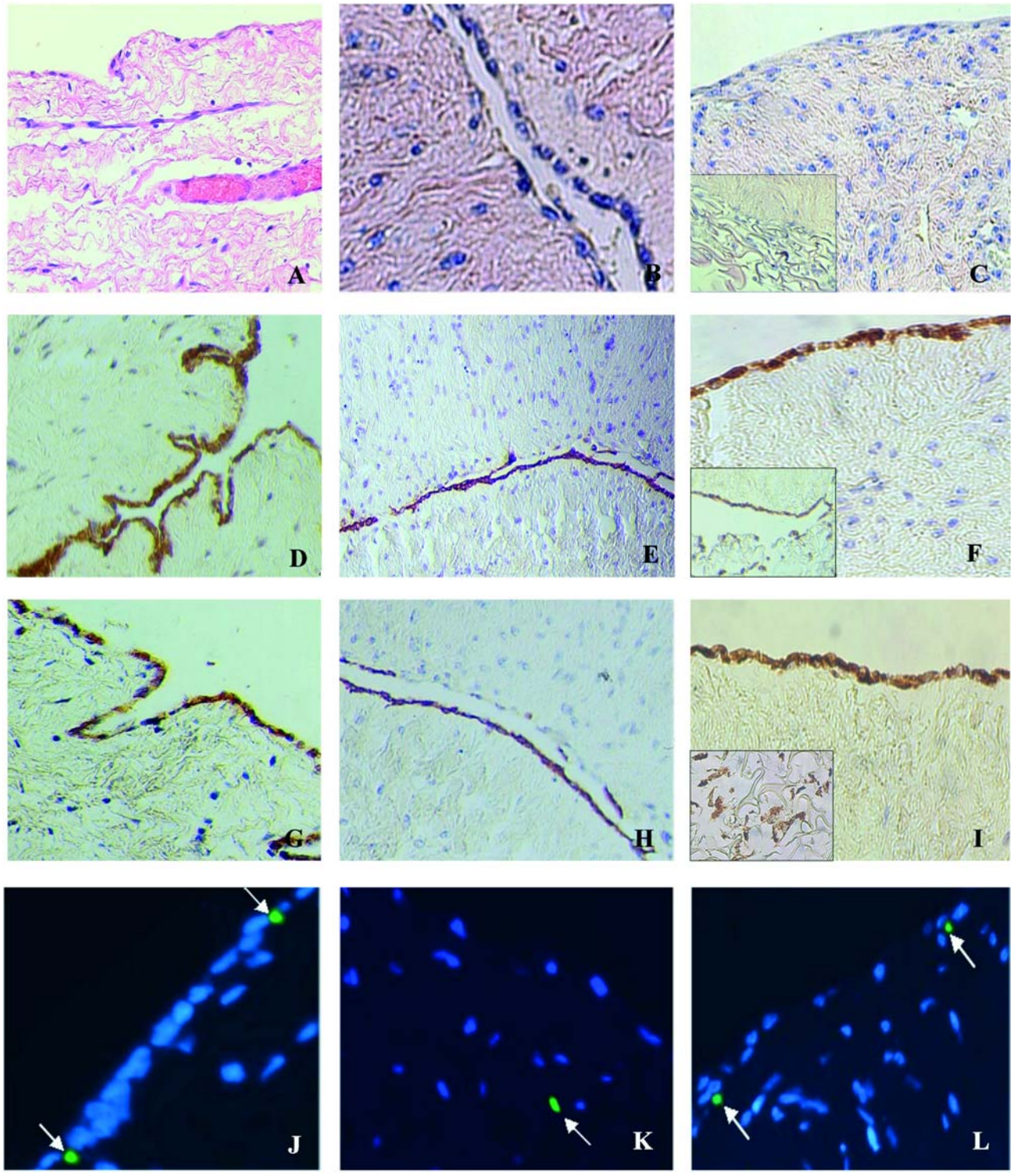

Fig. 1. Peritoneal-vaginal duct fragments in 3D-*in vitro* organotypic culture. Haematoxylin & eosin, prior to culture T0 (A), T1 (B) and T3 (C). Positive immunostaining for pan-cytokeratins (CKAE1-AE3) at T0 (D), T1 (E) and T2 (F). Staining for CK19 at T0 (G), T1 (H) and T2 (I). Nuclear positive staining for Ki-67, prior to culture T0 (J), T1 (K) and T2 (L).

cells migrating into the sponge. The percentage of positive iummuno-staining for HBME-1 was significantly lower in these cells. However, in some cultures we observed in T2 and T3 that HBME-1-positive cells displayed the tendency to line the cavities within the sponge (Fig. 2B and 2C).

Vimentin

Immunoreactivity for vimentin was detected in all structures present in evaluated tissue samples (Fig. 2D-F). However, although at T0 vimentin was expressed in a few mesothelial cells lining the fragment (Fig. 2D), at

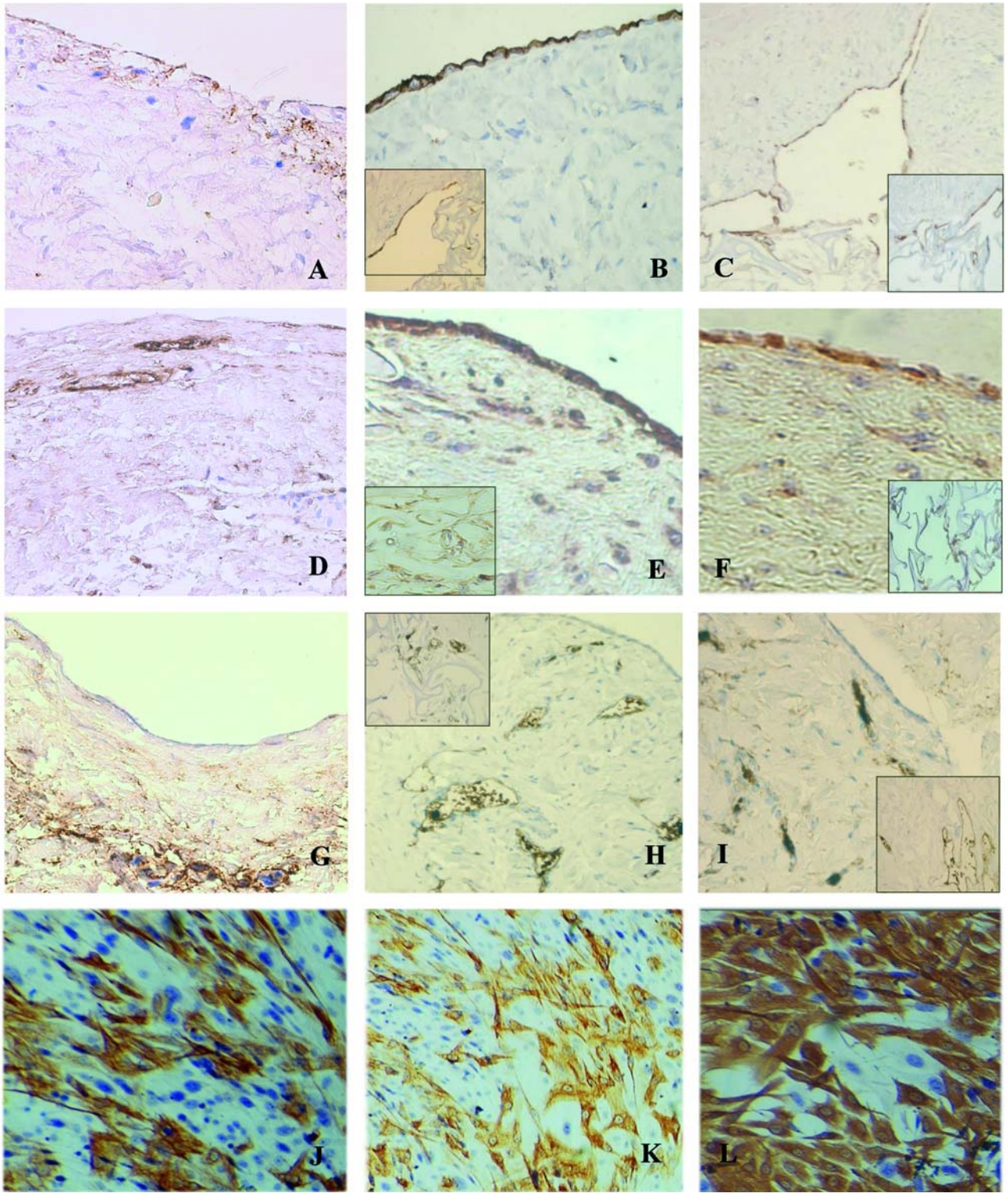

Fig. 2. Peritoneal-vaginal duct fragments in 3D-*in vitro* organotypic culture. Positive immunostaining for HBME-1 at T0 (A), T1 (B) and T2 (C). Staining for vimentin at T0 (D), T1 (E) and T2 (F). Staining for CD34 at T0 (G), T1 (H) and T2 (I). Immuno-staining performed on the cells immediately after isolation from the sponge at T3 and before culturing them on NIH.3T3 cells showed the expression of CK-19 (over 37% positive, 2J), pan-cytokeratins (over 70% CKAE1-AE3-positive, 2K), vimentin (over 90% positive, 2L)**.**

subsequent times of cultivation the entire mesothelial cell layer lining the surface of the fragment expressed vimentin (Fig. 2E and 2F). Flat mesothelial cells, mesenchymal cells inside the stroma and also the majority of spindle-shaped and stellate cells migrating into the sponge showed positive immunostaining for vimentin (Fig. 2E).

CD34

Endothelial cells lining the blood vessels in the stroma of tissue samples were CD34-positive (Fig 2G-I). Immunoreactivity for this marker was evident not only at T0, but also in cultured tissue fragments. Apart from blood vessels, CD34-positive, predominantly spindle-shaped cells were found dispersed inside the stroma of tissue samples (Fig 2G-I). Among the cells migrating into the sponge from cultured tissue fragments, only a few displayed CD34 immunoreactivity (Fig. 2H-I inserts); however, some of these CD34-positive cells formed small cavities within the sponge, resembling newly formed dilated capillaries (Fig. 2I).

p63

Detection of the p63 protein was entirely negative in all structures present in evaluated tissue samples at any time.

Isolation and clonal expansion of mesothelial progenitor cells in a two-dimensional monolayer in co-culture with NIH.3T3 fibroblasts as a source of feeder cells

Enzymatic digestion of small explants prior to culture or previously maintained on collagen sponges for 21 days in a 3D-organotypic culture was performed in an attempt to isolate and then expand *ex vivo* mesothelial progenitor cells using MIt-c-treated NIH.3T3 fibroblasts as a source of feeder cells providing a suitable microenvironment. The culture medium used was either $DMEM_{10}$ or $EpiLife_{10}$. Comparable results were obtained with these two media. Fig. 2J-L show the results of a representative experiment. A large fraction of cells isolated from sponges and fragments adhered to the plate. However, only a small fraction (about 1–5%) of the initial primary cultured mass could initiate colonies, in many cases forming mosaics of slowly enlarging colonies of tightly adherent epithelial-like cells. Only a limited proportion of adherent cells showed higher cloning efficiency and continuous proliferative capacity, both based on the size of the growing colonies, in addition to aborted colonies. We selected homogeneous, actively enlarging colonies that appeared to contain cells with high self-maintainance and a prolonged proliferation capacity; the cells of these colonies were pooled and when the cultured mass reached sub-confluence again, the cells were detached, dispersed by gentle pipetting and divided. These cells could be sub-cultivated by division at subconfluence for five passages. Colonies with a phenotype of epithelial-like cells decreased significantly within two successive cell passages, in favour of a spindle-shaped fibroblast-like morphology. This conclusion was supported by immunohistochemical analysis. Immunostaining performed on the cells immediately after isolation from the sponge at T3 and before culturing them on NIH.3T3 cells showed the expression of CK-19 (over 37% positive, Fig 2J), pan-cytokeratins (over 70% CKAE1-AE3-positive, Fig. 2K), vimentin (over 90% positive, Fig. 2L); all cells were constantly p63 negative. After the first passage, cultured cells showed a marked variation of the expression of the selected cellular markers: CKAE1-AE3 was positive in 39% cells, CK19-positive cells decreased to 15%, vimentin was positive in 95% cells, and p63 was never expressed. Finally, after the second passage, CKAE1-AE3 immunoreactivity was reduced to less than 10% cells, CK19 was undetectable, whereas vimentin-positive cells rose up to 98% and p63 was constantly negative.

Discussion

The aim of this work was to study the behaviour of the mesothelium of PVP fragments maintained in a 3D-organotypic culture on gelatin sponges kept submerged in a liquid medium and to estimate the response of the mesothelial progenitors in this natural microenvironment. The cultured tissue explants of PVP were evaluated using histological methods and by immunohistochemical analysis of various tissue markers at different times in culture. Similarly to other mesothelia, prior to culture the mesothelial layer of PVP showed intermediate characteristics of an epithelium (expression of CKAE1-AE3 and CK19) and of mesenchymal cells, by the expression of vimentin and HBME-1 (Mutsaers, 2004). These features were maintained over time in cells growing on the surface of cultured tissue explants and also in some cells migrating out of the fragment and infiltrating the sponge.

Initial phases of *in vitro* culture (T1) were characterized by regressive changes within the tissue fragments. These alterations manifested by focal detachment of mesothelial cells probably reflect tissue adaptations to the change of microenvironment from the organism to the culture. At T2, mesothelial cells spread around the cultured tissue fragments and appeared normally attached to the basal lamina. This was probably due to the capacity of mesothelium to substitute damaged parts and reconstitute a new and continuous lining of cells. This implies that mesothelial progenitor cells can survive and expand under our culture conditions and create new cellular layers in response to appropriate environmental signals. Such behaviour was found to be typical for epithelial cells and was observed in different types of cultured epithelia maintained in 3D-organotypic culture (Papini et al., 2003, 2005; Michelini et al., 2004, 2005). The underlying stroma was well conserved during the overall culture time; however, not all the vascular structures maintained their original 3D architecture *in vitro*, as shown by the finding that endothelial cells were often altered or detached into the lumen, at T1–T3. On the other hand, within the sponge we also observed the presence of small cavities formed

by CD34-positive cells, resembling newly formed dilated capillaries, suggesting that also these cells have self-renewal capacity *in vitro*.

Cells infiltrating into the sponge at T2 and especially at T3 displayed a more primitive phenotype. They were mainly spindle-shaped, fibroblast-like but co-expressed vimentin and cytokeratins. A similar situation also occurs *in vivo* in cases of tissue damage. Injured mesothelium regenerates *in vivo* from superficial mesothelial cells which proliferate, migrate, and finally cover the altered surface. The polygonal shape of regenerating mesothelial cells temporarily assumes a spindle-shaped fibroblastic morphology; subsequently, these cells migrate to the altered surface and become flattened once they have covered the surface (Whitaker and Papadimitriou, 1985; Floley-Comer et al., 2002). Phenotypic plasticity of mesothelial cells is also manifested in mesothelial tumours – mesotheliomas (Winstanley et al., 2006). Phenotypically primitive spindle cells displaying a fascicular arrangement predominate in sarcomatoid mesothelioma. Immunohistochemical markers of this tumour type include vimentin, actin and desmin. Cytokeratins are usually also positive. However, occasional sarcomatoid mesotheliomas display negative immunostaining for cytokeratins (Attanoos et al., 2000). Epithelioid mesothelioma, especially its more differentiated forms, shows remarkably bland epithelioid cytomorphology. Papillary, tubulo-papillary and adenomatoid (microcystic) patterns are predominant in this type of tumour. Neoplastic cells express cytokeratins (CK5/CK6), calretinin and Wilms tumour gene-1 (*WT1*) (Ordonez, 2005). Biphasic mesotheliomas are composed of both epithelioid and sarcomatoid patterns (Shimada et al., 2004).

Compared with 3D-organotypic cultures, cells recovered from the sponge at T3 by mild enzyme treatment and co-cultured in 2D with NIH.3T3 fibroblasts as a feeder cell layer behaved differently. In the primary culture, these cells showed double epithelial/mesenchymal phenotype (cytokeratin-positive, vimentin-positive), like cells growing within the sponge in the vicinity of the cultured tissue fragments. However, the proportion of vimentin immunoreactivity was higher (90% cells) than positive immunostaining for the cytokeratins (70% cells). After the first passage, cells cultured on the NIH.3T3 feeder layer maintained the mesenchymal phenotype (vimentin-positive), but expression of epithelial markers was again significantly reduced (the number of CKAE1-AE3- and CK19-positive cells was nearly half of the previous number). After the second passage, the cells lost CK19 expression completely and the epithelial phenotype was only confined to a small fraction of cells, while almost all the cells showed a mesenchymal phenotype. Substantial reduction of epithelial markers in co-culture with NIH.3T3 fibroblasts, in particular complete loss of CK19 (an epithelial progenitor cell marker), reflects the switch of differentiation from a double epithelioid-mesenchymal phenotype towards predominantly mesenchymal fibroblast-like cells. This could be due to the fact that cell-cell contacts with NIH.3T3 fibroblasts and soluble factors released by these cells cannot entirely substitute the conditions mediated by the natural stromal microenvironment which is present in cultured tissue explants of PVP. In support of this hypothesis, recent studies in which mesothelial cells have been shown to undergo an epithelial to mesenchymal transition and transformation into myofibroblasts and possibly smooth muscle cells strongly suggest the plastic nature of mesothelial cell progenitors (Yang et al., 2003). In our present work we have shown evidence that mesothelium in 3D-tissue explants of PVP maintains its double epithelial-mesenchymal phenotype. This may be due to the preservation of the original 3D-cytoarchitecture and the cellular heterogeneity present in the natural microenvironment (the *niche*) that allows progenitor mesothelial cells to self-renew and differentiate, maintaining their original phenotypic characteristics and function. Interestingly, despite the presence of cytokeratins, p63 was always negative in both 2D- and 3D-cultures of these PVP fragments. This is in agreement with previously published data showing that normal mesothelial tissue does not express p63 (Barbieri and Pietenpol, 2006).

In conclusion, the results of our present study demonstrate that the 3D-organotypic culture system can be successfully used to study mesothelial-stromal interactions and cell behaviour within intact natural PVP tissue explants. Additionally, the system provides a powerful tool for isolation and *ex vivo* expansion of mesothelial progenitor cells. It could be useful to further investigate the system in an attempt to employ cultured mesothelial progenitor cells in tissue engineering (Takazawa et al., 2005) or for drug testing.

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References

Asano, T., Takazawa, R., Yamato, M., Takagi, R., Imura, Y., Masuda, H., Kihara, K., Okano, T. (2006) Transplantation of an autologous mesothelial cell sheet prepared from tunica vaginalis prevents post-operative adhesions in a canine model. *Tissue Eng.* **12,** 2629-2637.

Attanoos, R. L., Dojcinov, S. D., Webb, R., Gibbs, A. R. (2000) Anti-mesothelial markers in sarcomatoid mesothelioma and other spindle cell neoplasms. *Histopathology* **37**, 224-231.

Barbieri, C. E., Pietenpol, J. A. (2006) p63 and epithelial biology. *Exp. Cell. Res.* **312,** 695-706.

Bedrossian, C. W. M. (1994) *Malignant Effusions: a Multimodal Approach to Cytologic Diagnosis.* Igaku-Shoin Medical Publishers Inc., New York, 211-243.

Bolen, J. W., Hammar, S. P., McNutt, M. A. (1986) Reactive and neoplastic serosal tissue. A light-microscopic, ultrastructural, and immunocytochemical study. *Am. J. Surg. Pathol.* **10,** 34-47.

Clarnette, T. D., Sugita, Y., Hutson, J. M. (1997) Genital anomalies in human and animal models reveal the mechanisms and hormones governing testicular descent. *Br. J. Urol.* **79**, 99-112.

Colbourne, C. M. Bolton, J. R., Mills, J. N. Whitaker, D., Yovich, J. V., Howell, J. M.. (1992) Mesothelioma in horses. *Aust. Vet. J.* **69**, 275-278.

Floley-Comer, A. J., Herrick, S. E., Al-Mishlab, T. Prele, C. M., Laurent, G. J., Mutsaers, S. E. (2002) Evidence for incorporation of free floating mesothelial cells as a mechanism of serosal healing. *J. Cell Sci.* **115,** 1383-1389.

Herrick, S. E., Mutsaers, S. E. (2004) Mesothelial progenitor cells and their potential in tissue engineering. *Int. J. Biochem. Cell Biol.* **36**, 621-642.

Hesseldahl, H., Larsen, J. F. (1969) Ultrastructure of human yolk sac: endoderm, mesenchyme, tubules and mesothelium *Am. J. Anat.* **126,** 315-335.

Lawrence, W. D., Whitaker, D., Sugimura, H., Cunha, G. R., Dickersin, G. R., Robboy, S. J. (1992) An ultrastructural study of the developing urogenital tract in early human fetuses. *Am. J. Obstet. Gynecol.* **167**, 185-93.

Michelini, M., Rosellini, A., Simoncini, T., Papini, S., Revoltella, R. P. (2004) A three-dimensional organotypic culture of the human uterine exocervix for studying mucosal epithelial differentiation and migrating leukocytes. *Differentiation* **72,** 138-149.

Michelini, M., Rosellini, A., Mandys, V., Simoncini, T., Revoltella, R. P. (2005) Cytoarchitecture modifications of the human uterine endocervical mucosa in long-term three-dimensional organotypic culture. *Pathol. Res. Pract.* **201,** 679-689.

Mutsaers, S. E. (2004) The mesothelial cells. *Int. J. Biochem. Cell Biol.* **36,** 9-16.

Ordonez, N. G. (2005) Immunohistochemical diagnosis of epithelioid mesothelioma: an update. *Arch. Pathol. Lab. Med.* **129,** 1407-1414.

Papini, S., Cecchetti, D., Campani, D., Fitzgerald, W., Grivel, J. C., Chen, S., Margolis, L., Revoltella, R. P. (2003) Isolation and clonal analysis of human epidermal keratinocyte stem cells in long-term culture. *Stem Cells* **21**, 481-494.

Papini, S., Rosellini, A., Campani, D., DeMatteis, A., Selli, C., Revoltella, R. P. (2004) Selective growth of epithelial basal cells from human prostate in a three-dimensional organ culture. *Prostate* **59**, 383-392.

Papini, S., Rosellini, A., Nardi, M., Giannarini, C., Revoltella, R. P. (2005) Selective growth and expansion of human corneal epithelial basal stem cells in a three-dimensionalorgan culture. *Differentiation* **73**, 61-68.

Peralta, S. A., Knudsen, K. A., Jaurand, M. C., Johnson, K. R., Wheelock, M. J., Klein-Szanto, A. J., Salazar, H. (1995) The differential expression of N-cadherin and E-cadherin distingueshes pleural mesotheliomas from lung adenocarcinoma. *Hum. Pathol.* **26**, 1363-1369.

Shimada, S., Ono, K., Suzuki, Y., Mori, N. (2004) Malignant mesothelioma of the tunica vaginalis testis: a case with a predominant sarcomatous component. *Pathol. Int.* **54**, 930-934.

Takazawa, R., Yamato, M., Kageyama, Y., Okano, T., Kihara, K. (2005) Mesothelial cell sheets cultured on fibrin gel prevent adhesion formation in an intestinal hernia model. *Tissue Eng.* **11**, 618-625.

Tiedemann, K. (1976) On the yolk sac of the cat. Endoderm and mesothelium. *Cell Tissue Res.* **173,** 109-127.

Tyler, W. S., Gillespie, J. R., Nowell, J. A. (1971) Modern functional morphology of the equine lung. *Equine Vet. J.* **3,** 84-94.

Wang, N. S. (1974) The regional difference of pleural mesothelial cells in rabbits. *Am. Rev. Respir. Dis.* **110**, 623-633.

Walts, A. E., Said, J. W., Shintaku, I. P., Sassoon, A. F., Banks-Schlegel, S. (1984) Keratins of different molecular weight in exfoliated mesothelial and adenocarcinoma cells – an aid to cell identification. *Am. J. Clin. Pathol.* **81**, 442-446.

Whitaker, D. Papadimitriou, J. (1985) Mesothelial healing: morphological and kinetic investigations. *J. Pathol*. **145,** 159-175.

Winstanley, A. M., Landon, G., Berney, D., Minhas, S., Fisher, C., Parkinson, M. C. (2006) The immunohistochemical profile of malignant mesotheliomas of the tunica vaginalis: a study of 20 cases. *Am. J. Surg. Pathol.* **30**, 1-6.

Yang, A. H., Chen, J. Y. Lin, L. K. (2003) Myofibroblastic conversion of mesothelial cells. *Kidney Int.* **63,** 1530-1539.