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Cell phenotype characterization in vascular organotypic culture

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An *in vitro* biocompatibility assessment test has been successfully applied to vascular prostheses. It consists of an organotypic culture of chick embryo vascular explant which preserves wall vessel cell interactions and enables cell growth and migration on biomaterial. This study is an attempt to identify the cell phenotype expressed by scanning electron microscopy, by immunostaining with specific monoclonal antibodies against smooth muscle cell (SMC) or von Willebrand factor (vWF), and by uptake of acetylated low-density lipoprotein. Early migrating cells had an endothelial-like phenotype independent of embryonic or adult vessel, veinous or arterial explants. SMC appeared labelled beyond the inner endothelial area. At the periphery, SMC displayed actin bundles specific to stationary phase. Adult cell cultures differed from embryonic cultures in that endothelial-like cells (EC) were more stable and increased their vWF labelling, whereas in embryonic cultures, some EC rounded up with subsequent detachment. Explant culture in liquid instead of agar medium activated this degenerative process suggesting the effect of diffusible chemotactic factors. Organotypic culture in agar medium provides an *in vitro* co-culture system of EC and SMC and enables further investigation of spatial and temporal evolution of vascular tissues in contact with various substrate.

1. Introduction

Synthetic vascular grafts are successfully employed in the replacement of stenosed or obstructed arteries. However, the use of small-calibre grafts (< 4 mm) is limited by their rapid occlusion due to anastomotic stenosis. These stenoses are often the consequence of an intense smooth muscle cell (SMC) proliferation leading to a pseudointimal thickening.

In order to improve the long-term patency of vascular grafts, research has been carried out to modify the prosthetic surface by plasma treatments [1] or by a coating step before implantation. Various proteins were then used in an attempt to promote endothelial cell (EC) spreading, migration and adhesion. Our work deals with the cytocompatibility of woven Dacron coated with albumin–gelatin crosslinked by carbodiimide. The biological properties of this coating were studied by using an organotypic culture, derived from the organ culture technique [2] as described below. This method presents the advantage of preserving the organ integrity and the cellular interactions. The vascular explant generated a cell layer which surrounded it, the cytocompatibility parameters of which were assessed: cell proliferation, migration and adhesion.

Although human organs could be used in organotypic culture, aortas and veins from chick embryos were preferred as vascular tissues. Results so obtained were consistent with other *in vivo* and *in vitro* techniques. Previous work reported that organotypic culture of adult rat and chick embryo vessel explants provided similar cytocompatibility assessment. They were corroborated by the patency of vascular prostheses implantation in dog [3, 4]. Recently, a comparative study established that organotypic and dispersed cell cultures yielded complementary informations relative to the vascular prostheses cytocompatibility [5]. In both cases, culture substratum regulated the extracellular matrix secretion and modulated the cell phenotype expression. While dispersed cell cultures provided homogeneous population, organotypic cultures mainly differed by their ability to release cells from different tissues of the vascular wall. Thus, injured endothelium of explants in close contact with biomaterial may liberate EC and/or SMC; the later may change from a contractile to a synthetic form in response to specific factors [6].

The purpose of this work was to follow the phenotype evolution of the growing cell layers from embryonic vessel explants incubated either in liquid (explant culture) or in agar (organotypic culture) nutrient medium. Adult vessel explants were cultured to provide differentiated cells available for immunostaining controls. All the cultures were grown on albumin–gelatin membrane designed for coating Dacron.

2. Materials and methods

2.1. Crosslinking of protein membranes

Fraction V bovine albumin (Sigma) and pigskin gelatin (Rousselot) were covalently crosslinked using

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carbodiimide. Albumin–gelatin membranes (AG) were obtained by mixing albumin 20% with gelatin 10% (1 v/2v) in PBS 0.2 M pH 4.75. The AG membranes were air dried, post-reticulated by adding 0.2 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (Sigma) extemporaneously diluted in PBS pH 4.75 and set over a sterile glass plate then detached and thoroughly rinsed in sterile PBS pH 7.42. Small square pieces were cut for organotypic and explant cultures.

2.2. Culture techniques

The organotypic culture consisted of artery or vein fragments of 14 day old chick embryos and of 1 month old chicken, respectively, layered onto agar medium, the endothelial side facing the AG membrane as previously described [5]. Dispersed cells were harvested by trypsin-EDTA (0.125%–0.10%) treatment of the cell layers surrounding the explants previously grown for 7 days in liquid nutrient containing Medium 199 + RPMI (v/v) + 20% foetal calf serum, L-glutamin 2 mM. Cells were seeded onto membranes set in Petri dishes and fed with the same medium added with EC growth supplement (Sigma) 50 µg/ml and heparin (Sigma) 90 µg/ml.

2.3. Cell phenotypic characterization

2.3.1. Scanning electron microscopy

Cell layers were fixed in 3% glutaraldehyde in Rembaum buffer (pH 7.4), dehydrated in graded alcohols, critical-point dried from CO_2 (Polaron Instrument Inc.) and examined using a Jeol (model JSM 840) scanning electron microscope.

2.3.2. Silver staining

The silver nitrate staining was performed as previously described [7]. This coloration revealed the intercellular junctions in a monolayer epithelium-like tissue. Chick fibroblast cultures were used as a negative control.

2.3.3. Double indirect immunofluorescence assays

Cell cultures previously fixed in absolute methanol at – 20°C, were successively incubated for 30 min with rabbit polyclonal anti-human von Willebrand factor (vWF) antibody (Sigma), anti-rabbit antibody labelled with rhodamine (Sigma), mouse monoclonal anti α-smooth muscle actin antibody (Sigma) and finally with fluorescein conjugaed anti-mouse immunoglobulin antibody (Sigma). A washing step with PBS + 1% BSA was intercalated between each immunoreaction. The samples were examined with a Leitz microscope equipped with epifluorescence illumination.

Immunostaining specificity was controlled by the following tests: (a) substitution of the primary antibody by nonimmune serum from rabbit or mouse; (b) exclusion of the primary antibody; (c) immunostaining of chick embryo SMC and fibroblast with the anti vWF and anti α-smooth muscle actin antibodies, respectively.

2.3.4. Uptake of acetylated LDL

Endothelial cells were characterized using the selective uptake of acetylated low-density lipoprotein (Ac-LDL). Cell cultures were incubated with 10 µg/ml acetylated lipoprotein labelled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, Inc, Cambridge MA), at 37 °C in culture medium for 4 h as previously described [8].

3. Results

3.1. Cell morphology

Initial cell characterization was realized by scanning electron microscopy (SEM). Cell layers from embryo explants incubated for 7 days on agar medium exhibited two different circular areas. In zone A, which enclosed the explant, cells had a characteristic appearance that distinguished them from other cell types. They were in apposed contact with each other and appeared large and polygonal (Fig. 1a). The external area B, larger than the internal, constituted elongated and preferentially oriented cells, suggesting a strong migration. These cells grew in a multilayered "hills and valleys" pattern, which is a typical feature of SMC cultures (Fig. 1b). The presence of two distinct cell areas was confirmed by the silver staining: the cells in A were closely apposed and their limits were strongly

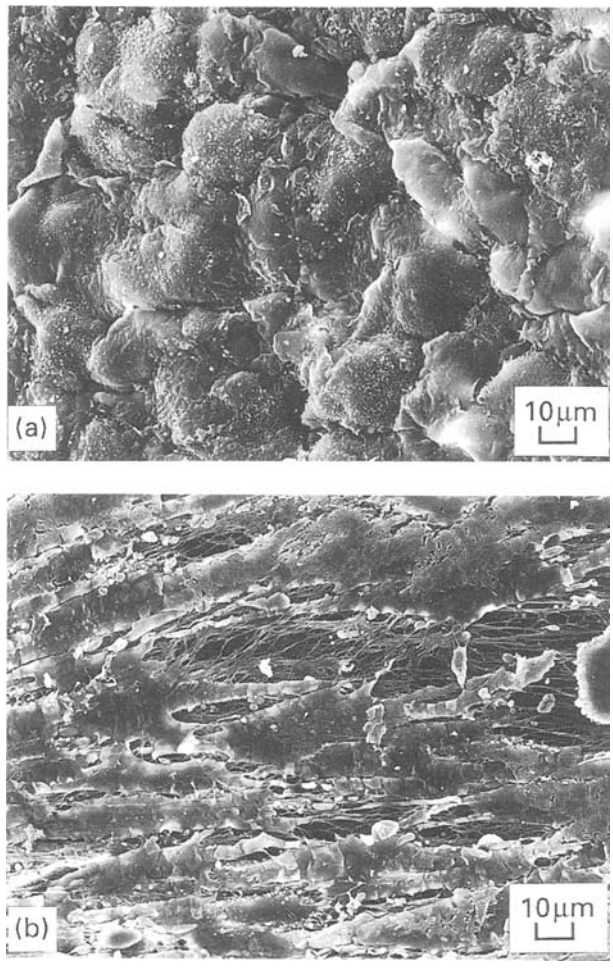

Figure 1 Scanning electronmicrographs showing cell layers of embryo explants incubated on agar medium: (a) zone A near the explant, (b) intermediate zone B.

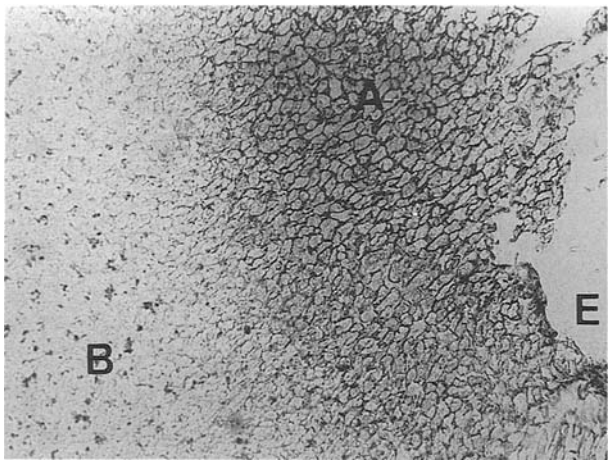

Figure 2 Silver-stained culture of embryo explant on agar medium. The area A close to the explant E, detached before staining, contains distinctly labelled cells whereas the external layer B is not shadowed (× 125).

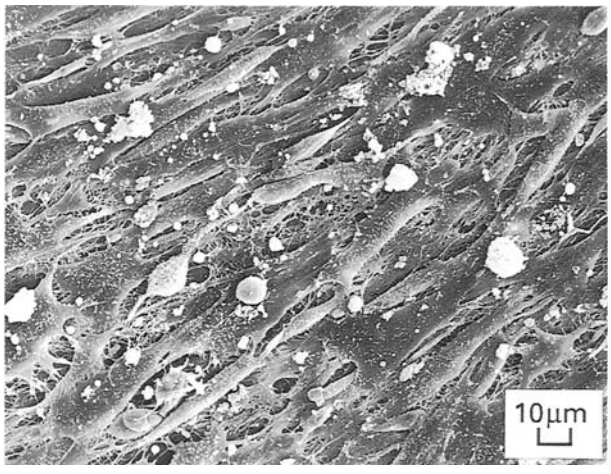

Figure 3 Scanning electronmicrograph of cell layers of embryo explant grown in liquid medium.

contrasted, whereas the intercellular junctions in B were barely visible (Fig. 2). Cell layers from vascular explants grown in liquid medium seemed to be constituted of a homogeneous area of well-elongated cells (Fig. 3). Similarly, vascular dispersed cell cultures displayed homogeneous elongated cell shapes which formed an oriented cell layer.

3.2. Cytoimmunological staining

Immunofluorescence study performed on organotypic culture samples with the anti vWF antibody showed numerous bright fluorescent dots in the cytoplasm of the A area cells, whereas anti α-smooth muscle antibody did not show fluorescence. Cells constituted a homogeneously marked layer (Fig. 4a). The cells belonging to area B exhibited opposite staining results: they did not express the vWF antigen, but fluorescent bundles pointed up the cytoskeleton components. The single cells scattered at the culture periphery exhibited an organized actin bundle (Fig. 4b), described as a characteristic feature of stationary cells [9].

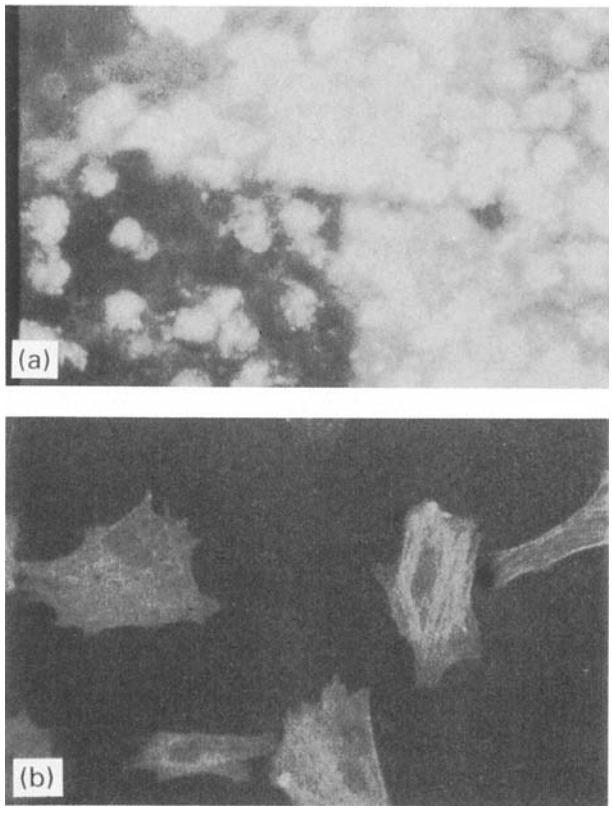

Figure 4 Indirect immunofluorescence analysis of the cell phenotype in organotypic culture of chick embryo vessel explants: (a) rhodamine staining of cells belonging to the area A which positively reacted to anti vWF antibody; (b) fluorescent staining of cells at the culture boundary, by anti α-smooth muscle actin antibody (× 500).

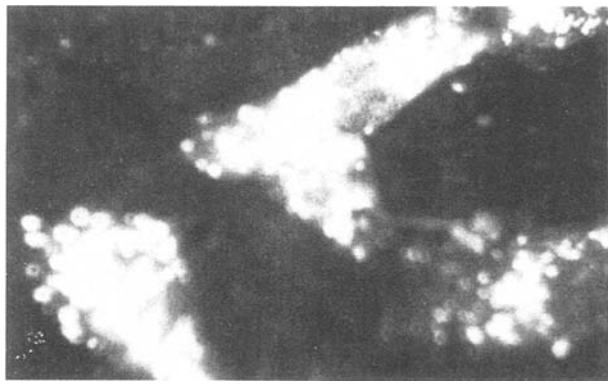

Figure 5 Rhodamine staining of early cells positively labelled with anti vWF antibody in organotypic culture of chick embryo vessel explants (× 1250).

The differentiation state of embryonic organ cultures was further analysed in comparison with vascular explant cultures from 1 month old adult chicken which could be specifically labelled. Immunofluorescence stainings were the same in both cultures. Moreover, no qualitative difference could be observed between the cell layer of arterial and veinous explants independent of embryonic or adult origin. Furthermore the double immunostaining performed after 20 h incubation revealed the presence of vWF antigen in the cytoplasm of the early cells emerging from the embryonic explant (Fig. 5). In these 14 day cultures, the cells located in A, positively probed by the anti vWF

antibody, often rounded up and their density decreased. Nevertheless in a few samples of adult explant cultures, a homogeneous layer of strongly labelled cells persisted (Fig. 6).

Cell layers from vascular explants, grown for 7 days in liquid nutrient medium, did not show different areas but a homogeneous culture of smooth muscle-like cells appeared, although the first migrating cells reacted positively with anti vWF antibody (Fig. 7a, b).

Double immunostaining of dispersed cell cultures revealed different labelling depending on their origin. Cultures from embryo vessels presented two cell phenotypes: cells positively probed by anti-α smooth muscle actin antibody and not by anti vWF antibody and cells positively labelled by the two antibodies (Fig. 8a, b). Both cell types were equally distributed and had similar morphological appearance. Cell cultures proceeding from adult vessels showed only positive immunostaining for α-smooth muscle actin.

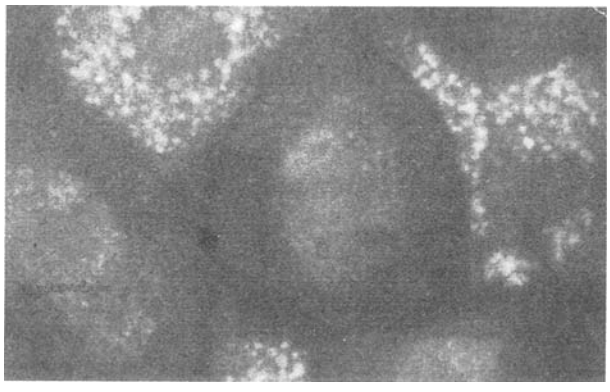

Figure 6 Late fluorescent anti vWF labelling of A area cells in organotypic culture of adult chick vessel explants (× 1250).

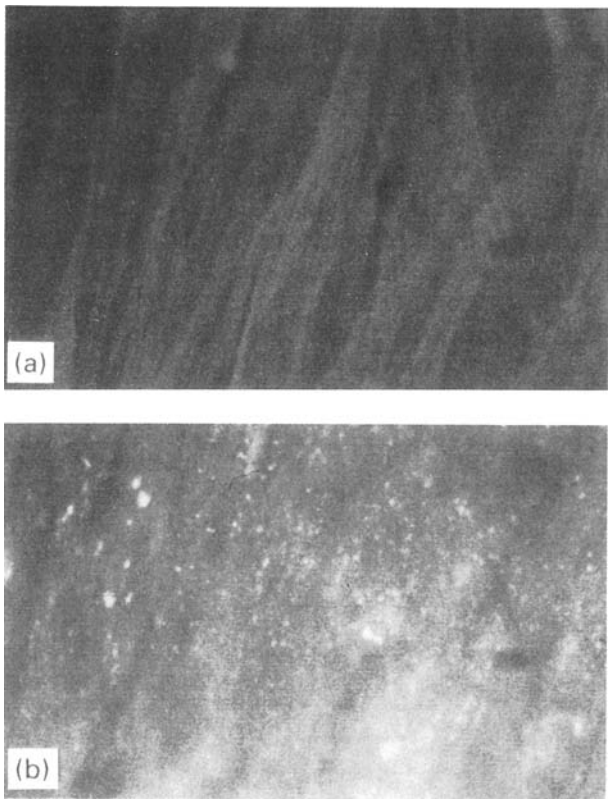

Figure 7 Double indirect immunostaining of explant culture of chick embryo vessel: (a) fluorescent anti α-smooth muscle actin staining, (b) non specific anti vWF staining (× 1250).

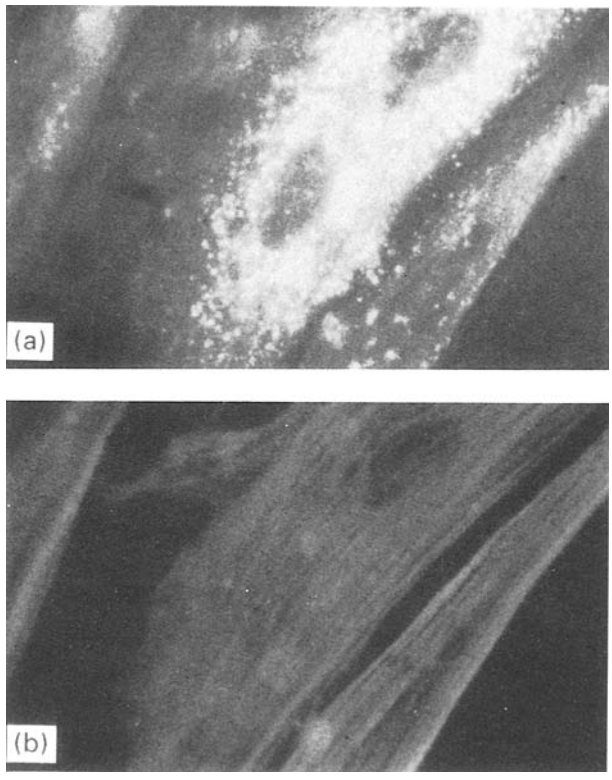

Figure 8 Double indirect immunostaining of dispersed cell culture previously harvested from a chick embryo explant culture: (a) rhodamine staining of vWF antigen, (b) fluorescent labelling of α-smooth muscle actin (× 1250).

3.3. Uptake of acetylated LDL

In organotypic culture of embryonic explants, Ac-LDL was incorporated only by cells in area A. Up to 4 days the staining was uniform and concerned all the apposed cells (Fig. 9a). After this lag, a scattered cell population was probed (Fig. 9b). In explant cultures, fed with liquid medium, as in dispersed cell cultures, cells were unable to incorporate the Ac-LDL.

4. Discussion

While the use of chick embryo organs offers numerous advantages because they react towards biomaterials like some adult tissues and display adaptation possibilities to new culture conditions, the phenotypic differentiation of the growing cells may be restrictive. The four reported tests are intended to investigate three cell features: morphology, cytoimmunological expression and metabolic function. The control assays confirmed the staining specificity as previously reported [10]. Indeed, Ac-LDL and human vWF were described as useful markers to identify chick embryonic EC both *in situ* and in cell culture from aortic arches of embryonic chicken.

Organotypic cultures carried out with embryonic and adult vascular explants were all concordant. The inner circled area would be superficially constituted by EC: they showed a flattened, polygonal cell shape, and reacted positively with both the anti vWF antibody

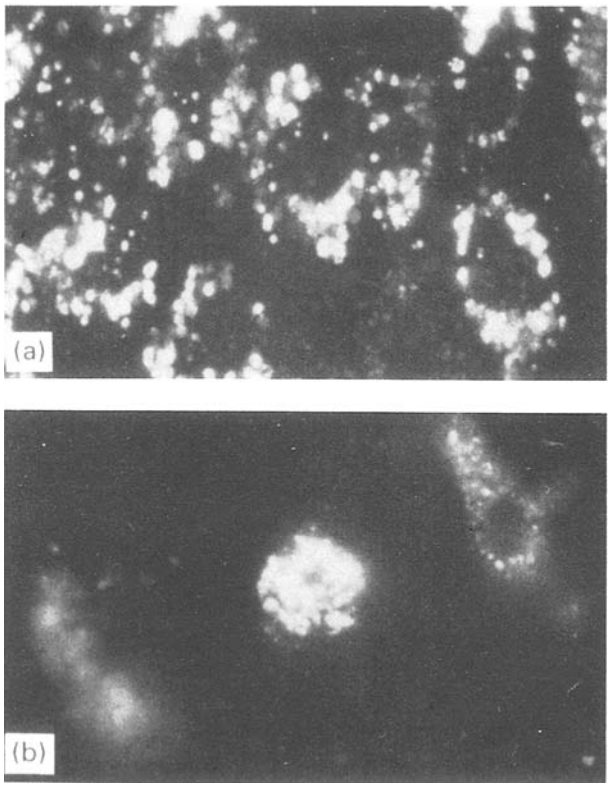

Figure 9 Uptake of fluorescent Ac-LDL in organotypic culture of chick embryo vessel explants: (a) by day 3, all the cells were positively labelled; (b) by day 6, labelled cells were scattered (× 1250).

and the Ac-LDL, but not with the anti α-smooth muscle actin antibody. The results of the three stainings confirmed the presence of SMC in the external area. Thus, these culture conditions would allow the vascular explants to generate a coculture. The EC were the first growing cells from the explants (Fig. 5), whatever the vessel nature (arteries or veins), or the vessel origin (embryonic or adult). However, up to 6 days, embryonic EC population seems to degenerate as shown by the results of the metabolic staining, probably due to culture conditions, whereas in a few samples of adult explant cultures, we note an accumulation of vWF into the cytoplasm of the inner circled area cells.

Although the first cells emerging from explants incubated in liquid medium had an endothelial labelling, the endothelial phenotype vanishes beyond day 3 and the cell layer appears to be constituted only of SMC, as revealed by the three staining reactions. This difference with regard to the culture performed in agar medium seems to be independent of medium renewing since it can also be observed in the absence of cell feeding. Then the EC phenotype fate may be correlated with the diffusion of cell secreted compounds in the nutrient medium.

The results of the three staining reactions corroborated that the 7 day dispersed cell cultures from adult vascular explants contained only SMC. This is not surprising since the primary culture, that is the explant culture in liquid nutrient, was essentially constituted of these cells. In contrast, the three tests performed in the presence of the dispersed cell cultures from embryonic vascular explants give different results. Whereas the observations provided by SEM and the test of Ac-LDL uptake pointed out the presence of homogeneous SMC population, the double immunostaining revealed various phenotypes. This apparent cell diversity may correspond to an unspecific staining of embryonic cells, although these passaged cells proceeded from a homogeneous SMC culture.

Therefore the organotypic culture of vascular explants in agar medium allows the expression of two cell phenotypes and this co-culture enables temporal and spatial evolution (areas A and B). The dynamic interaction of intimal SMC and EC had been previously studied over 1 month in a porcine aortic organ culture system [11, 12]. Their organ culture differed essentially from ours by the absence of a close contact between endothelium and culture substratum which enabled, in our conditions, cell outgrowth. Their experiments showed that proliferation of pre-existing intimal SMC was enhanced by soluble mitogens released from EC injury and/or death. Kinetic measurements indicated a direct relation between EC turnover and intimal SMC growth rate. They also observed a few cells rounding up with subsequent detachment. We found a similar process in the organotypic culture but the loss of EC was enhanced in liquid medium culture conditions. Such a temporal evolution in the course of primary culture might depend on a number of factors, including the presence of chemotactic and mitogenic molecules. It is well known that vascular wall injuries release growth regulatory agents in the local environment. Basic fibroblast growth factor (bFGF), a potent stimulator of EC proliferation and other mesenchymally derived cells, such as fibroblasts and SMC, is released from these cells when they are injured or dying [13, 14]. Furthermore, rat arterial SMC in primary cultures or after a lesion increase their platelet derived growth factor (PDGF) synthesis and secretion and this growth factor acts via autocrine or paracrine mechanisms, by chemotactic and mitogenic effects on these muscle cells [15]. Bovine vascular EC have been shown to secrete PDGF-like molecules [16], but the lack of PDGF receptor make them unresponsive to these growth factors [17]. Thus, the successive way out of EC and SMC may be a consequence of growth promoting effects of bFGF and PDGF. Afterwards, the presence of two cell types generates an *in vitro* coculture system and allows exchange of diffusible substances. In fact, the latent form of transforming growth factor beta (TGF-β), secreted by bovine EC and SMC monocultures, may be activated by this cell co-culture [18–20]. The active form of TGF-β has a self-regulating effect which may stimulate or inhibit SMC proliferation depending on the experimental conditions [21]. It also displays an inhibitory effect on EC growth [22] such as TNF-α.

Whether the growth regulation of chick vascular EC and SMC in co-culture proceeds the same way as in human and other mammalian cell co-cultures is somewhat speculative. The mechanism by which the cell phenotype shifts from an early EC to a late SMC after a delay may involve cell relationship by way of chemotactic regulators. It could not be excluded that SMC

were released from the media without interacting with EC, SMC outgrowth from mesoderm requiring longer incubation time than EC outgrowth from the endothelium which is in close contact with the substratum.

5. Conclusions

Attempting to mimic the *in vivo* situation by respecting the reciprocal interactions which occur between EC and their immediate vascular environment, we have carried out an *in vitro* organ culture system to study the cell behaviour of damaged vascular wall in direct contact with Dacron precoated prostheses. The organotypic culture of vascular explants in agar medium allows the expression of two cell phenotypes, EC and SMC and this co-culture enables spatial and temporal evolution. Modification of culture to a liquid medium and multiple passages favour the smooth muscle phenotype.

Acknowledgements

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