PROLIFERATION OF ISOLATED HUMAN ENDOTHELIAL CELLS ON DIFFERENT COLLAGEN SUPPORTS

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In this study, we searched for a dependable system for *in vitro* colonization of various collagen supports with human endothelial cells and determined their biocompatibility and proliferation.
Support collagen matrices and membranes were employed to investigate the colonizing capacity of human endothelial cell line, EA hy 926. The biocompatible supports employed were: Pancol (collagen matrix, high resorptive capacity), Amatcol (collagen matrix, low resorptive capacity), Plascol (membrane collagen on textile insertion net) and collagen membranes (control). The attachment of cells on biomaterials was monitored by fluorescence microscopy, the cell structure by transmission electron microscopy and the cell proliferation was assessed spectrofluorimetrically. All collagen supports sustained, endothelial cells proliferation. Fluorescence microscopy and proliferative tests showed different colonization rates on collagen biocompatible supports. The higher level of colonization was obtained on Pancoll. Ultrastructural examination of the EA hy 926 grown on collagen matrices revealed the presence of a specific organelles and a large number of secondary lysosomes. Cells grown on collagen membranes were similar in structure with control cells.
The study of the biointegration capacitiy of endothelial cells on collagen supports revealed (1) no significant differences between Plascol and control membrane, (2) Amatcol represent a better substrate for cell colonization in comparison with Pancol.

Key words: Collagen supports; Endothelial cells; Biocompatibility.

INTRODUCTION

The production of engineered tissues is an emerging field which holds promise to improve current medical therapies. Tissue engineering involves seeding a three dimensional scaffold with cells, expanding the cell population, and then implanting the engineered tissue construct *in vivo*. The natural biopolymers provide biologically specific signals for molecular interaction with the delivered cells and interact specifically with cells of the target tissue [1-4]. Protein based extracellular matrix gels, such as fibrin, collagen, or a mixture of collagen, laminin, and other proteins forming Matrigel, are commonly used to create two-, or three-dimensional cell culture substrates of controlled stiffness[5]. Because collagen is well established as a safe and effective biomaterial, it was one of the first to be used in biomatrices development. Collagen has been identified as a mildly antigenic fibrous protein; it has been considered suitable for use as a biomaterial. In addition, collagen, a biological macromolecule, has been found to have low immunogenicity, absorbability, an adjustable biodegradation rate, and a good biocompatibility. Thus, collagen is considered as a candidate material to combine with water-soluble synthetic polymers to produce hybrid systems with various properties, compositions, and forms [6].

It is known that type I collagen is the most abundant collagen of the human body[1]. It is also

present in scar tissue, the end product when tissue heals by repair, it is found in tendons, the endomysium of myofibrils and the organic part of bone.

Adhesion to type I collagen can affect cell morphology, differentiation, and cell cycle progression, depending on its structure[7]. When polymerized into fibrils, as it is mostly found in vivo, type I collagen inhibits growth of a number of cell types including smooth muscle cells[8], melanoma cells[9], glomerular epithelial cells[10], and hepatocytes [11, 12], whereas adhesion to monomeric collagen stimulates cell cycle progression under similar culture conditions. Unlike loss of adhesion, however, which blocks cell cycle progression and also induces apoptosis, adhesion to collagen gels promotes survival and increases differentiated function [13, 14].

In the human body several processes do not have efficient regenerative capabilities, if any at all. As an alternative for current medical treatments for damaged or substitution of lost tissue, new biopolymers were developed. Appropriate scaffolding material is needed to provide support and promote cell regeneration. As collagen proteins are a major structural element in numerous body's tissues and organs, collagen fibers are a logical choice for scaffolds.

An essential element in graft procedure is the blood supply. The prevention of implant failure caused by hypoxia and following infection is still a challenge. There are different therapeutic strategies to enhance angiogenesis and wound healing in diseased or injured tissues: (i) implantation of modified bioactive materials, (ii) implantation of cells and (iii) implantation of biohybrids that are assembles of cells and scaffolds[15-17]. In general, cell-based tissue engineering provides a successful treatment in wound healing disorders.

Endothelial cells are the major cell type of the microvasculature. The response of these cells to a biomaterial is of great importance to the success of the biomaterial. Neovascularization is an important step in wound healing and tissue repairing. This study examines the *in vitro* interaction of human endothelial cells with collagen membranes and matrices.

MATERIALS AND METHODS

Chemicals used for cell cultures were obtained from Sigma (USA) and those used for electron microscopy were form Polysciences (USA), except sodium sulphate and lead citrate, which were obtained from Merck (Germany), and tannic acid from Mallinckrodt. Tissue culture flasks were from Costar (Cambridge, MA, USA).

Collagen supports

The support matrices and membranes are made of collagen type I, extracted from bovine derma. Collagen membranes were obtained by free drawing procedure at 25º C temperature and matrices by lyophilization (freeze-dried procedure). Two types of matrices were employed: Pancol (collagen matrix, very resorptive) and Amatcol (collagen matrix, low resorptive), and two types of membranes: Plascol (membrane collagen on textile insertion net) and collagen membrane (control).

Collagen supports colonization

For *in vitro* colonization we used the endothelial cell line EA hy 926 (human aortic endothelial cells) grown in DMEM supplemented with 4,5‰ glucose medium, 10% fetal bovine serum, and sodium selenite 20 μg/l, 30 mg/l ascorbic acid, and antibiotics (100 U/l penicillin, 100 U/l streptomycin, 50 U/l neomycin). Collagen biomaterials were sterilized with 70% ethanol for 24 hours. The collagen membranes and matrices were conditioned in the same culture medium for 24 hours and then inoculated with EA hy 926 endothelial cells (50 000 cells/ml). Cells were maintained in culture at 37°C in incubators with 5% CO_2 in air (v/v), and relative humidity over 95%. All experiments were done after 1 week of culture.

Hoechst staining

The cells were cultured on collagen supports for one week, washed in PBS, fixed in 2% paraformaldehyde for one hour, and then cryoprotected. After washing in phosphate tampon (PB: 0,2M Na_2HPO_4, and 0,2 M NaH_2PO_4) pH 7.2, the specimens were kept in a solution of PB 0.1 M+5% sucrose over night at 4°C. In the next day, the probes were immersed in PB containing 5%, 10%, 20% and 50% glycerin at 4°C for 15 minutes, 1 hour, 10 hours and 1 hour, respectively. Specimens were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome; the thickness of the sections were 4–6 μm. The cryosections were washed with PBS for 15 minutes, stained with Hoechst 33258 for 15 minutes (a specific DNA staining), washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence and a filter G1-B; the micrographs were captured with a Sony DSC-S75 Digital Camera.

DNA estimation

A simple and rapid assay for quantitative DNA determination in cell culture was used[18]. The cells grown on collagen sponges cubes (1 mm^3) and collagen membranes squares (1 mm^2) for 1 week were placed in 24 wells plates, and then the cells were broken by freezing in liquid nitrogen and rapid thawing (original method). Then Hoechst 33258 dye (200 μl/well), dissolved in 10 mM Tris, 1mM EDTA, 2 M NaCl in double distiled water (TNE buffer, pH 7,4, 10 μg/ml) were added. DNA standard curves were constructed using human leucocytes DNA. The measurements were performed on a TECAN 96-well plate reader (excitation at 350 nm; emission at 460 nm). All experiments were done on 8 probes for every collagen supports.

Transmission electron microscopy (TEM) was preformed according to Jinga *et al* [19]. Throughout the procedure, all buffer solutions were used at pH 7.4 and the osmolality of 300 mOsm was employed. Briefly, the cells grown on collagen supports were washed twice with 75 mM sodium cacodylate buffer supplemented with 3% (w/v) sucrose (SCB) and fixed for one hour and 30 min in 2.5% glutaraldehyde in SCB and again washed twice with SCB. Then, the cultures were postfixed in 2% (w/v) osmium tetraoxide in 150 mM sodium cacodylate buffer (CB) for 90 min, at 4°C, then washed twice for 2 min in cacodylate buffer followed by mordanting in 1% (w/v) tannic acid for 10 min and two rinses in 1% (w/v) sodium sulphate in 100 mM cacodylate buffer [20]. The cultures were then rapidly dehydrated in increasing concentrations of ethanol: 70% (v/v) (1 × 5 min), 95% (1 × 5 min) and 100% (3 × 5 min) and embedded in 1:1 (v/v) Epon 812: ethanol for 30 min and then in Epon 812 (100%). The resin was polymerized at room temperature for 1 hour, and then at 37°C for 4 hours and at 55° C for two days. Thin sections obtained on a Reichert OmU3 ultramicrotome were stained with 7.5% (w/v) uranyl acetate for 10 min with 0.4% (w/v) lead citrate for 1.5 min, and examined by transmission electron microscopy employing a Philips EM 201C and Philips EM 400 (Holland).

Biomaterials colonization was monitored by fluorescence microscopy, transmission electron microscopy and spectrofluorimetric DNA quantification.

RESULTS AND DISCUSSIONS

Endothelial cell colonization on collagen supports

Employing Hoechst staining we have found that collagen matrices and membranes sustained cellular growth; however the colonization was made at different ratios. Collagen matrices *i.e.*, Pancol and Amatcol, allowed three-dimensional growth (at the surface and inside of the matrices) within the structural macropores, whereas collagen membranes, that presented micro- and nano-structures accepted cellular growth only on the surface (Figs.1, 2).

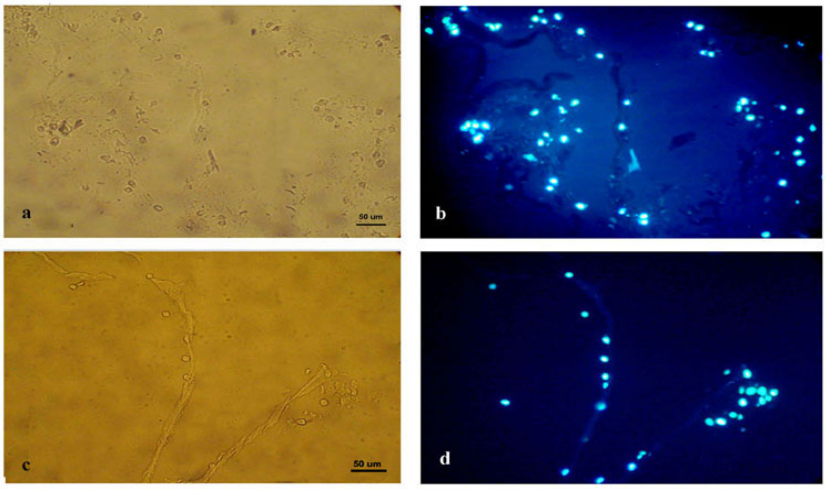

Fig. 1. Endothelial cells grown on Pancol (a, b) and Amatcol (c, d) collagen matrices.

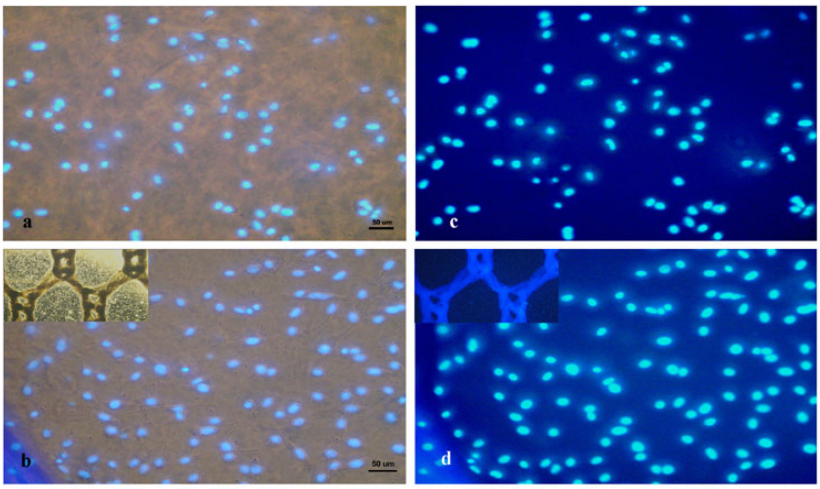

Fig. 2. Endothelial cells grown on collagen membranes (a, c) and Plascol (b, d), inset – endothelial cells grown on Plascol (ob. × 4).

Cellular proliferation differed on various materials. We compared two varieties of collagen matrices and membranes. In the case of collagen matrices, the higher level of colonization was obtained on Amatcol (Fig. 3a). As for membranes, the proliferation was better on pure collagen membrane compared to Plascol (Fig. 3b).

All tested scaffolds sustained endothelial cells proliferation. Collagen supports and membranes induced different colonization rates as revealed by fluorescence microscopy and Hoechst nuclear staining. Collagen matrices allowed penetration of cells due to macro-, and micro-pores. The matrices reticulation grade affected hydrophilic properties and water absorption; as showed in Figure 3, the endothelial cells growth and proliferation was augmented on Amatcol as compared to Pancol. Moreover, collagen membranes showed a higher level of colonization as compared with matrices. Micro-, and nano-structures of collagen membranes sustained the endothelial cells growth only on their surfaces.

Ultrastructure of endothelial cells grown on collagen membranes and matrices

Upon interaction with collagen biomaterials, the structural aspect of endothelial cells was not significantly modified (Fig. 4). Ultrastructural examination of the endothelial cells integrated in the biocompatible collagen supports revealed the presence of a well developed rough endoplasmic reticulum, and numerous free ribosomes. Cells grown on Pancoll presented a large number of secondary lysosomes (Fig. 4 a, b), compared with Amatcol which exhibited fewer secondary lysosomes (Fig. 4 c, d). The same applies for the cells grown on collagen membranes (Fig. 5 a, b, c, d) that were similar in structure with the control cells (Fig. 6).

The above data demonstrated that the collagen membranes provide a better support for endothelial cells proliferation than collagen matrices. Ultra-structural aspects of cells cultured and integrated into collagen biomaterials were different in matrices and in membranes. In cells grown on collagen matrices the number of secondary lysosomes is increased, indicating that these supports affect the activity of endothelial cells. The presence of secondary lysosomes may be explained by a deficient diffusion of oxygen to the cells in the 3D configuration of the matrices, as compared to the cells grown on the surfaces of membranes. The ultrastructural aspects of endothelial cells are another indication that the collagen membranes were more biocompatible than collagen matrices. A possible explanation of this finding is that in the drying procedure, the collagen fibrils from gel solution become arranged in a similar way with the *in situ* collagen fibrils.

CONCLUSIONS

The capacity of various collagen supports for endothelial cells biointegration may vary according to the type of collagen employed: (1) for collagen membranes, no differences were observed between Plascol and control collagen membrane whereas (2) Amatcol represents a better substrate for colonization in comparison with Pancol. The results recommend the differentiated use of Amatcol and collagen membranes in wounds healing and as part of prosthesis reconstruct for cardio- vascular surgery.

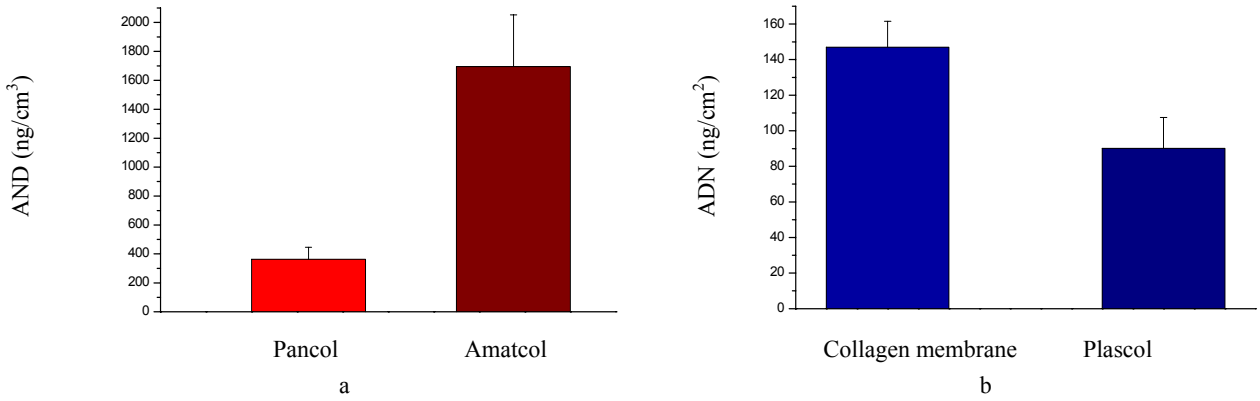

Fig. 3. Endothelial cells proliferation on different collagen supports as assessed by DNA quantification: a. collagen matrices; b. collagen membranes.

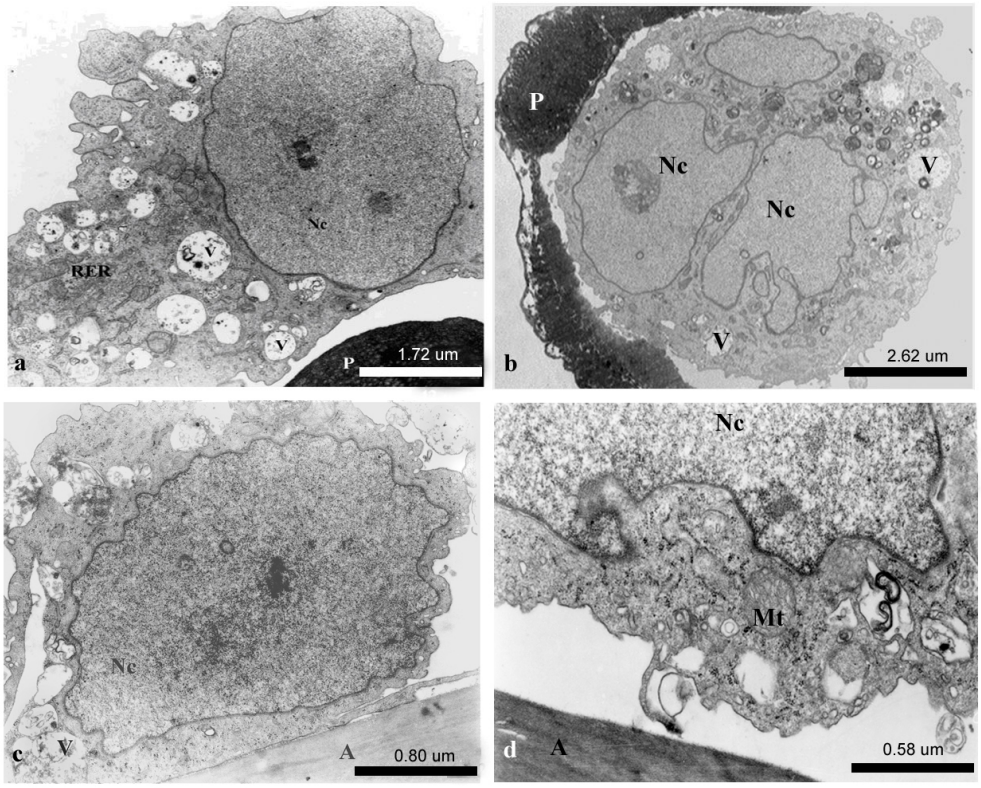

Fig. 4. Ultrastructural aspects of endothelial cells grown on collagen matrices a, b – endothelial cells grown on Pancol (P); c, d – endothelial cells grown on Amatcol (A) Nc – nucleu, V – vacuoles, RER – rough endoplasmic reticulum; Mt–mitochondria.

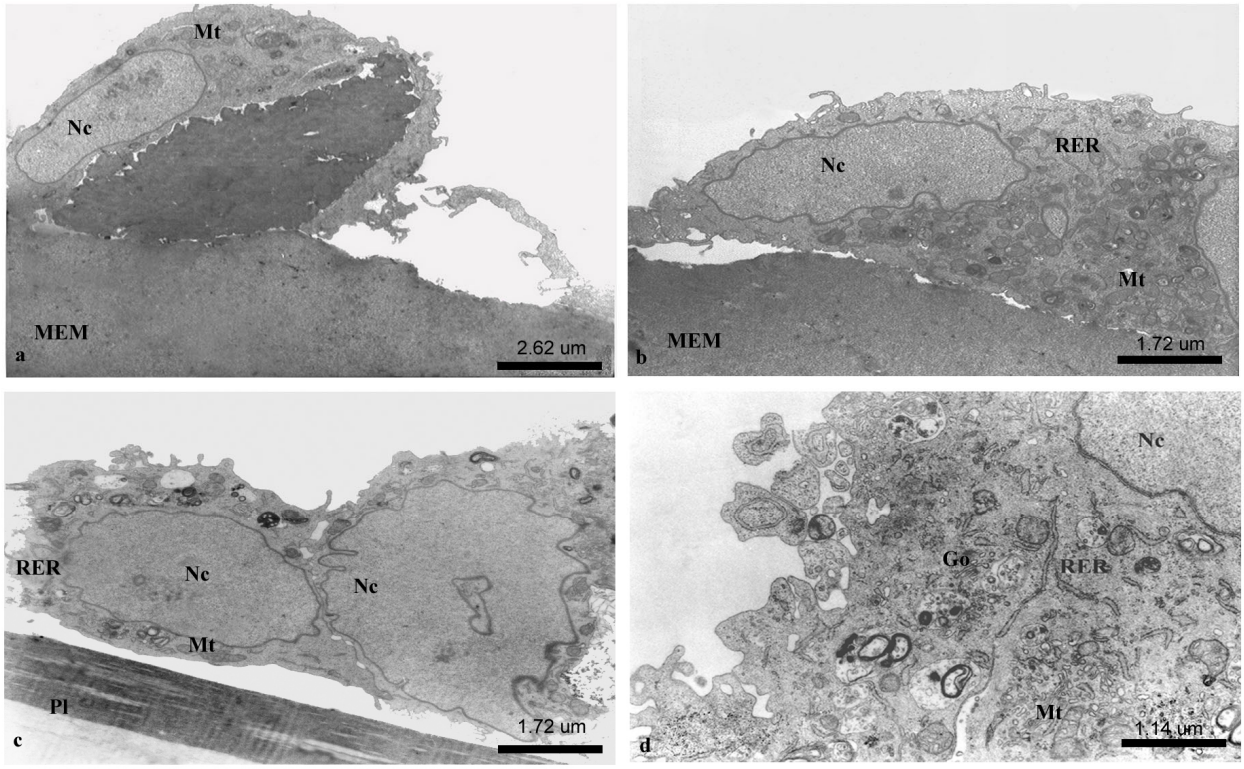

Fig. 5. Ultrastructural aspects of endothelial cells grown on collagen membranes (a, b) or on Plascol (c, d); Nc- nuclueus; RER – rough endoplasmic reticulum; Mt–mitochondria; Go – Golgi apparatus; MEM – collagen membrane; Pl – Plascol.

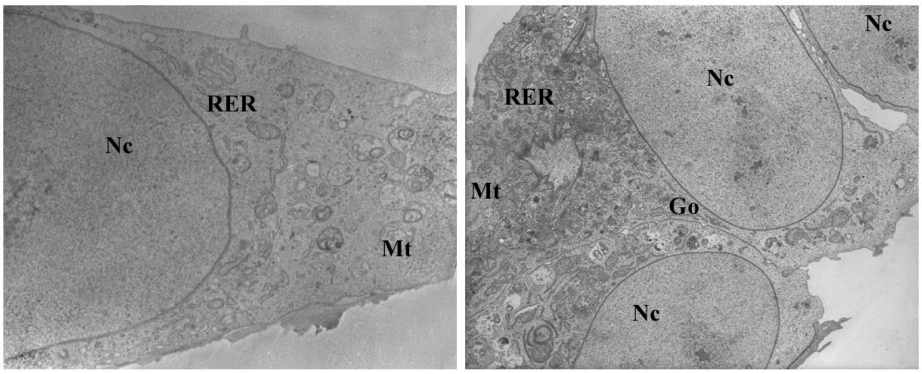

Fig. 6. Ultrastructural aspects of endothelial cells grown on borosilicate glass surface (control), Nc – nuclueus; RER – rough endoplasmic reticulum; Mt–mitochondria, Go – Golgi apparatus.

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