

Endothelial stem cells on a synthetic surface?

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Palabras clave: Prótesis arterial, micropatronización, células tronco endoteliales, endotelialización.

RESUMEN

Hay una fuerte evidencia de la presencia de una población heterogénea de células progenitoras endoteliales (CPEs) en la circulación sanguínea, y en las células endoteliales que son utilizadas en las superficies sintéticas. Estos datos sugieren que las CPEs pueden participar no sólo en mantener la integridad y función del endotelio vascular, si no también en la angiogénesis. El descubrimiento de las CPEs, ha generado interés en el campo de la ingeniería tisular. Las prótesis arteriales sintéticas (PAS) de diámetro pequeño tienen una vida media muy corta. La duración de las prótesis esta relacionada con la trombosis, ésto debido a una falta de adhesión entre las células endoteliales y el bionanomaterial sintético. Sin embargo, la presencia de células tronco endoteliales (CTEs), durante el proceso de adhesión es desconocida. Aquí, mostramos la presencia de células CD34+ en una superficie de politetrafluoroetileno (PTFE). La superficie de esos filmes de PTFE fue tratada con emisiones radiofrecuenciadas de plasma amonio, conjugado con sulfosucinimidil 4-(N-maleidometil)ciclohexano-1-carboxilato (S-SMCC), y micropatronizado con las secuencia de péptidos CGRGDS y CWQPPRARI, con el propósito de promover la adhesión de células endoteliales aórticas bovinas (CEABs) en la superficie de PTFE. Nuestros resultados demuestran la adhesión de CEABs en la superficie PTFE diseñada, pero

también soportan la evidencia que CTEs podrían estar presentes en las superficies de PTFE y promover la endotelialización. Creemos que al determinar las señalizaciones promotoras de las CTEs, nos permitiría usarlas como un futuro tratamiento en las prótesis arteriales sintéticas.

Key words: Arterial prostheses, Micropatterning, endothelial stem cell, endothelialisation.

ABSTRACT

There is a strong evidence for the presence of a heterogeneous population of endothelial progenitor cells (EPCs) in the sanguine circulation and in the endothelial cells (ECs) that are used on synthetic surface. These data suggest that EPCs may participate not only in maintaining the integrity and function of vascular endothelium but also in forming new blood vessels. The discovery of EPCs in tandem with emerging concepts in stem cell biology has generated interest and excitement in many fields such as tissue engineering. Synthetic arterial prostheses (SAPs) of small diameter have a very short lifespan. The durability of prostheses is related to the thrombo formation due to a lack of adhesion between endothelial cells and the synthetic bionanomaterial. However, the presence of endothelial stem cells (ESCs) during this adhesion process is unknown. Here we show the presence of CD34+ cells on polytetrafluoroethylene (PTFE) surface. The surface of these PTFE films was treated with radiofrequency glow discharge ammonia plasma, conjugated with sulfosuccinimidyl

4-(N-maleidomethyl) cyclohexane-1-carboxylate (S-SMCC), and micropatterned with peptides sequences CGRGDS and CWQPPRARI, with the purpose of promoting the adhesion of bovine aortic endothelial cells (BAECs) on PTFE surface. Our results demonstrate the adhesion of BAECs on

patterned PTFE. Our findings support evidence suggesting that ESCs could be present on the PTFE surface and to promote the endothelialisation. We believe that determining the promoter signalling of ESCs would allow manipulating them with the objective of using them as future treatment in SAPs.

INTRODUCTION

Synthetic arterial prostheses have today a frequent use but the durability of prostheses of small diameter is short due to the formation of thrombosis and stenosis. This durability can be increased if the host body recreates the endothelium on the internal surface of the prosthesis (Salaciski *et al.*, 2001; Tiwari *et al.*, 2002). Adhesion of cells to the extracellular matrix (ECM) influences growth, differentiation, proliferation, migration and communication of cells. One well-understood mechanism in which components of the basement membrane modulate cell adhesion is the activation of $\alpha_5\beta_1$ integrin receptors which attach to the tripeptidyl sequence Arg-Gly-Asp (RGD) binding site of fibronectin (Chiquet *et al.*, 1996; Mousa & Cheresh., 1997). Previous works have demonstrated that RGD peptides grafted on the polytetrafluoroethylene (PTFE or Teflon) surfaces promote *in vitro* adhesion of endothelial cells (Mooradian *et al.*, 1993; Huebsch *et al.*, 1995; Gauvreau & Laroche, 2005).

It is accepted that bone marrow stem cells can generate an heterogeneous population of cells. However, Ingram *et al.*, (2005) have identified a complete hierarchy of EPCs in the vessel wall, providing a new framework for classification of cells supporting endopoiesis akin to that previously established for haematopoiesis. Stem cells and progenitors cells play an important role not only in maintaining the function and integrity of vascular endothelium but also in angiogenesis (Doyle & Caplice, 2005). However, a few is known about the presence of stem cells characteristic of the

endothelium on a treated PTFE surface that is to say in a synthetic arterial prosthesis.

With the purpose of analyzing the endothelialisation process of a synthetic surface with the use of a new micropatterning method and the presence of endothelial stem cells, we performed a three steps process for grafting peptides on the inert PTFE surface. First, an ammonia plasma reaction allows the incorporation of amino groups on the PTFE surface. Secondly, the conjugation of these amino groups with the sulfosuccinimidyl 4-(N-maleidomethyl) cyclohexane-1-carboxylate (S-SMCC) introduces maleimide functionalities on the activated PTFE surface. The last step is a covalent reaction between S-SMCC-modified surfaces and the cystein amino acid included at the NH_2 extremity of the peptide sequence. It is, at this step of the process that the micropatterns are introduced. The micropatterning principle that we developed use the properties of aerosols (Baek *et al.*, 1996; Akhtar & Yule, 1999; Beck & Watkins, 2002) (Fig. 1). A spray nozzle forms an aerosol with a small amount of peptide solution projected toward the surface to pattern. The droplets of the spray then adsorb on the surface permitting the covalent reaction between the maleimide functionalities and the peptides. Each adsorbed droplet creates a microscopic round spot on the surface. The maleimide functionalities are known to be stable for many hours permitting many successive treatments of the surface with different aerosol patterns and finishing with a uniform background by plunging the surface in the peptide solution. This innovative approach is particularly well adapted for

micropatterning large three-dimensional biomaterials that do not need geometric patterns or the presence of well defined arrays (Frankel, 1992; Chen *et al.*, 1997; 1998; Kanda *et al.*, 2004; Liu *et al.*, 2004). Contrary to standard micropatterning, the process we developed let the patterns position be random. This principle is based on the fact that *in*

vivo, cell does not grow in squares or perfect straight lines. The treated surfaces are then seeded with bovine aortic endothelial cells (BAECs). Our purpose with this research is to promote the endothelialisation process with the use of a new micropatterning method and analyse the presence of endothelial stem cells in these surfaces.

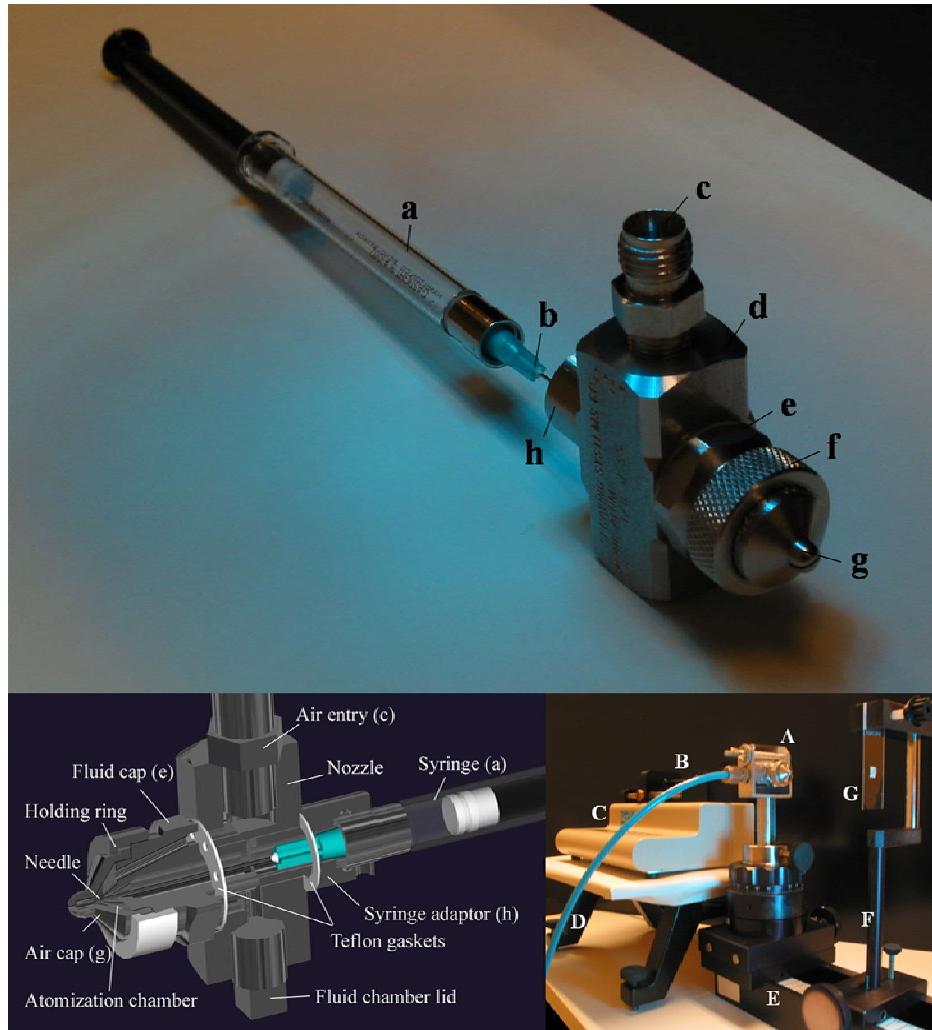


Fig. 1. Description of the micropatterning apparatus. The atomization nozzle permits to fraction the solution in small droplets. It is composed of: a) 2.5 Hamilton syringe, b) 32 gauge medical needle with sharp end polished straight, c) Air entry, d) Nozzle body e) Fluid cap, f) Holding ring, g) Air cap and h) Syringe adaptor (Top), Computerized inside view of the atomization nozzle, Teflon Gaskets with holes permit the air to reach the atomization chamber without leakage. Solution is brought in the atomization through a medical grade needle. The original fluid chamber has been replaced with a 2.5 ml Hamilton syringe. (Bottom left), Complete montage of the apparatus including the spray nozzle A), 2.5 ml Hamilton syringe B), diffusion pump C), air line D), rail permitting to vary distance between atomization nozzle and sample E), sample holder F), sample G) and Bottom right.

MATERIAL AND METHODS

Micropatterning apparatus.

An air atomizing nozzle (SU14SS, Spraying Systems Co., Wheaton, IL) was mounted on a goniometer rail. The volume and flow rate of the solution were controlled using a Cole-Parmer micropump (780100C, Vernon Hills, IL). Air pressure regulator (FESTO, HE ¼ S-B, 0702, Mtl, CA.) allows modifying the air pressure. A medical grade needle (23 gauge, 38 mm length) with the sharp end cut, straight and polished, was plugged in a 2.5 cc Hamilton syringe (Gastight No 1002, Hamilton Company, Reno, NV) and fitted through the fluid cap allowing only a small amount of liquid to reach the atomizing chamber. A sample holder designed for glass microscope slides 1.5-mm thick or less, on which the PTFE samples were fixed (PTFE films 0.25 mm thick were acquired from Goodfellow, Huntingdon, England), was also mounted on the rail allowing the samples to move closer or further from the spray nozzle. The characterization of the surfaces was made using image analysis software (CLEMEX Vision, Longueuil, QC). Patterns were first characterized using aqueous solutions of glycerine (7% v/v), coloured with a blue dye and sprayed directly on the glass slides. Patterns of peptides were made by spraying a solution of CGRGDS and CWQPPRARI in a 1 µM/ml solution on PTFE samples.

Ammonia Plasma Treatment.

The 4 cm x 1.6 cm PTFE films were treated at a radio frequency (rf) of 13.56 MHz and a power of 20 W and a pressure of 300 mTorr for 60 s using high purity ammonia gas. The ammonia rf plasma treatment and system apparatus were previously described elsewhere (Gauvreau *et al.*, 2004). The PTFE samples were rolled inside in a 6 mm cylindrical plasma chamber. The whole sample was then cut into 18 pieces of 0.35 cm² for the subsequent grafting procedures. Verification of the surface modification was done using a PHI 5600-ci spectrometer (Physical Electronics, den Prairie, MN,

USA) using an X-ray source at a power of 400 W XPS spectra was used to record the spectra. For every measure the detection was performed at a take-off angle of 45°.

Surface functionalization.

The ammonia plasma treated film was immediately immersed and shake in 2 ml of a solution of 2 mg/ml of S-SMCC (Sigma, Milwaukee, WI, USA) in PBS 0.2 M at pH 7.4 solution for 2 h in polypropylene tube. This reaction was driven in darkness to avoid UV degradation of the S-SMCC substrate and in a nitrogen environment to avoid oxidation of the reactive amino groups of the fresh plasma treated surface. The characterization of this reaction has been presented elsewhere (Gauvreau *et al.*, 2004). Samples were then vortex-rinsed with deionised water 6 times for 30 sec each time in the polypropylene tube to remove any non-covalently bound molecule.

Peptide solution.

Plasma S-SMCC treated surfaces were then sprayed with the peptides solution. The peptide sequences CGRGDS and CWQPPRARI (were synthesized by Service de synthèse de peptides de l'Est du Québec, CHUQ, Québec, QC, Canada) were dissolved in solutions of 2 µM peptide/ml of PBS 0.2 M at pH 7.4 (Sigma, Milwaukee, WI, USA) containing 7.5% glycerol (Laboratory MAT, Montreal, Canada) were prepared. Glycerol was added to adjust the peptide solution viscosity and to prevent rapid evaporation of the solution when the peptides were used. Unpatterned peptide pre-treated surfaces were obtained by complete immersion in the solution. The patterns for cell growth support were obtained with pressures of 69, 138, 248 kPa (10, 20 and 36 psi). Reacting time was 3 h in humidity controlled room to avoid drying or condensation on the samples. Samples were finally washed with flowing deionised water for 30 sec 6 times each.

Endothelial Cell Culture.

BAECs (Cambrex Bio Science Walkersville Inc., MD, USA) were grown in a 75 cm² tissue culture-treated polystyrene flasks. They were seeded at 40 000 cells/well in 48 wells plate for 10 min adhesion tests. For proliferation test, after 10 min of adhesion the surface was gently immersed in PBS to remove unattached cells, then immersed in high glucose Dulbecco's modified Eagle media with 10% veal serum (Hyclone, Utah, USA) and 100 µg/ml penicillin G, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B (Invitrogen) for 3, 24 and 72 h.

Fixation and staining.

The culture medium was aspirated from the wells and cells were fixed for 30 min at room temperature with 3.7% formaldehyde in PBS. The formaldehyde solution was then removed from the wells, and cells were washed three times with PBS. Cell permeability was increased with a solution of PBS with 3% BSA and 0.1% saponin (Sigma, Milwaukee, WI, USA) for 1 h 30 min at room temperature. This solution was removed and cells were stained using DAPI (0.3 µM in PBS, Sigma, Milwaukee, WI, USA) and rhodamine-phalloidin (6.6 µM in PBS, Sigma, Milwaukee, WI, USA) and maintained in the 37° C incubator for 1 h. Then cells were washed 2 times with PBS, and we added CD34 (1:500, eBioscience, San Diego, CA, USA). Cells were then washed six times with PBS and 0.05% Tween 20 before surfaces were fixed between blade and lamella to be observed under the microscope.

Microscopy and Image Analysis.

Microscopic pictures of BAECs seeded surface were taken using a Nikon E800 confocal microscope equipped with a Hamatsu Orca ER

digital camera (Nikon Canada, Mississauga ON, CAN). Pictures were analyzed using CLEMEX Vision software (Longueuil, QC, Canada). Nine pictures per surface sample were taken and analyzed. Three-dimensional reconstructions were obtained with Huygens Essential 3.1.0 (Scientific Volume Imaging B.V. Hilversum, The Netherlands).

RESULTS AND DISCUSSION

We showed that patterns obtained with the developed technique have a strong effect on BAECs grown on patterned PTFE (Fig. 2). These films were treated with ammonia plasma and were immediately immersed in S-SMCC. This reaction is complete and permits the introduction of maleimido groups on the PTFE surface to subsequently conjugate peptides through a sulfhydryl containing N-terminal Cysteine residue. Surfaces with spots of an average diameter of 10±3 µm had the best endothelialisation and surfaces with spots of CGRGDS over a background of CWQPPRARI were more effective than the opposite surface containing spots of CWQPPRARI over a background of CGRGDS. Surfaces with spots of an average of 100±3 µm killed almost all the cells. Spots with an average of 20±3 µm did not show a significant difference with unpatterned surfaces. On the other hand, BAECs are cells obtained from the aortic endothelium and an interesting possibility is the presence of stem cells among the BAECs population. To verify this hypothesis, we utilized the CD34, commonly used as a stem cell marker with the purpose of detecting stem cells on a synthetic surface (Chiu *et al.*, 2005; Covas *et al.*, 2005; Lu *et al.*, 2005; Jaffredo *et al.*, 2005). We demonstrated the presence CD34+ cells (0.020 ± 0.009%) among the population of BAECs after 72 h of surface colonization (Fig. 3, Fig. 4).

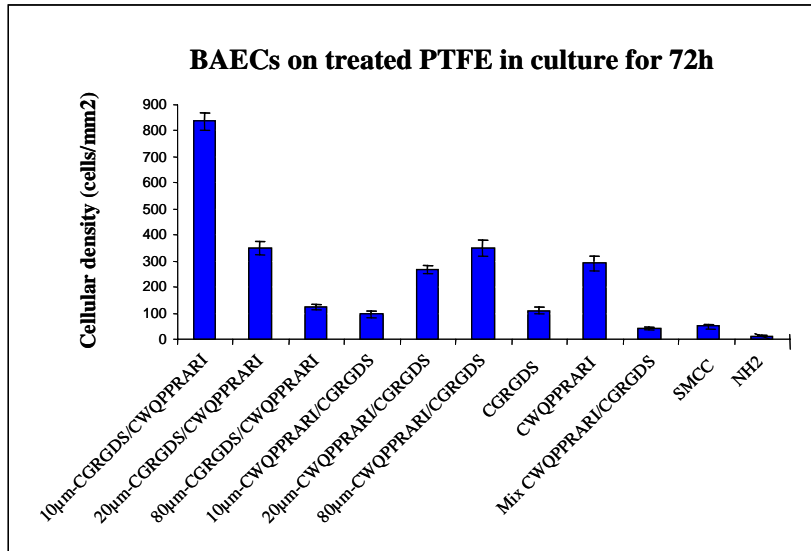


Fig. 2. Density of cells on different treated PTFE surfaces. X axis definitions correspond to: diameter of the spots in microns- peptides in the spots / peptide around the spots.

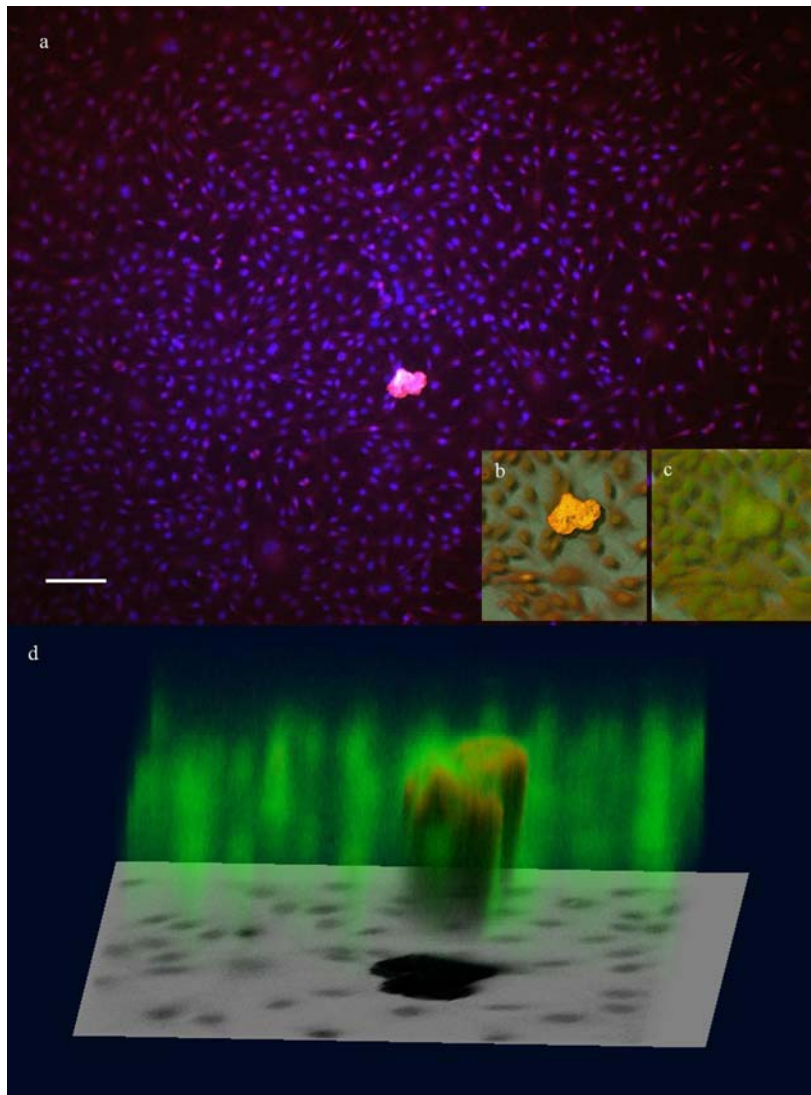


Fig. 3. a) Immunofluorescence for CD34 (yellow), DAPI (cell nuclei, blue) and Rhodamin-Phalloidin (actin, red) on Teflon surface when the endothelialisation process is established. Scale bar 100µm. b) Expression of CD34 (of the same cell as (a) in a confocal three dimensions image. c) Same cell (a, b) with rhodamin phalloidin, actin marker, highlighted in dark red forming a web over the treated PTFE surface. d, Side view image in three dimensions of the expression of CD34 of the same cell (a,b,c).

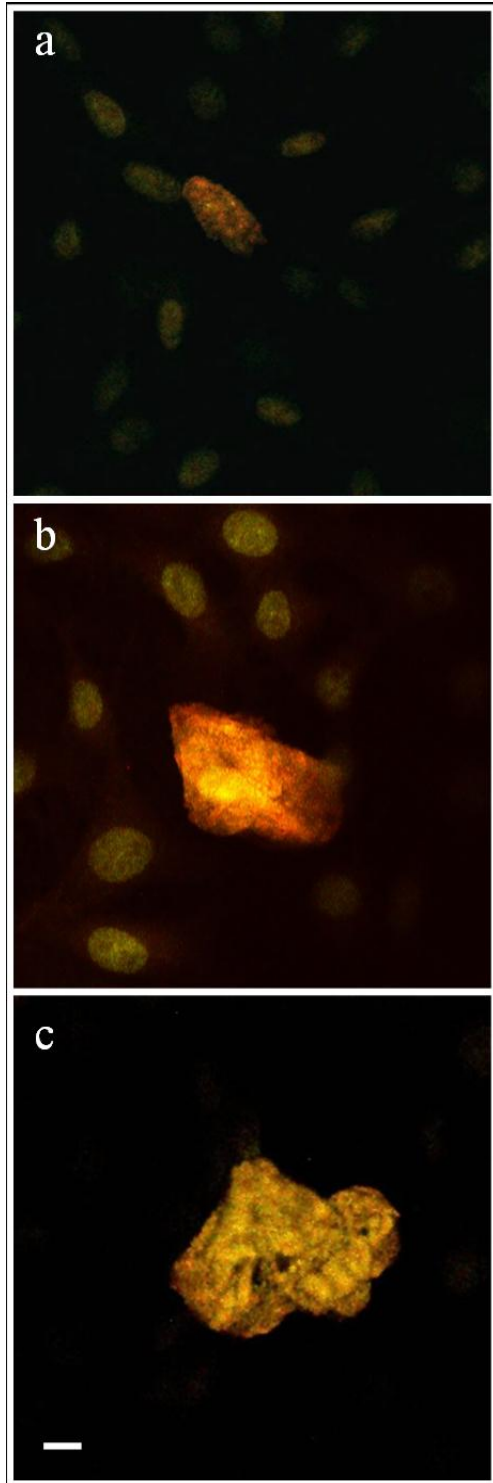


Fig. 4. Different morphologies of observed CD34+ cells on synthetic surface. a-c CD34+ expression. Confocal photomicrographs showing CD34+ (a, b, c gold), DAPI (a, b, green) and Rhodamine-Phalloidin (b, red). Scale bar, 10 μ m.

For a long time, the processes that favor the endothelialisation using umbilical vascular endothelial cells or aortic endothelial cells have been studied and considered a homogeneous population of mature ECs. But recent evidences demonstrate that ECs are a heterogeneous population of cells (Walenta *et al.*, 2005; Ingram *et al.*, 2005; Jaffredo *et al.*, 2005). In our study, we used aortic endothelial cells and our findings suggest that stem cells may be present in the aortic endothelium. At the moment, a specific marker for endothelial stem cell does not exist and it is not possible to determine whether the (CD34+) cells identified in Fig. 3 and 4 are stem cells or progenitor cells. However, we demonstrated the presence of CD34+ cells when the endothelialisation process is established on a synthetic surface. The eventual presence of stem cells could play a major role in the endothelialisation of the native artery and synthetic prostheses. We think that these cells have the capacity to promote the endothelialisation of artificial artery grafts. We have a suitable model to continue studying the behavior of the endothelial stem cells to promote the endothelialisation phenomena in synthetic arterial prostheses since very little is known about the behavior of endothelial stem cells on a treated PTFE surface.

CONCLUSIONS

Our work demonstrated the presence of viable CD34+ cells on a treated Teflon synthetic surface. It also demonstrated that a new method for micropatterning of a surface could enhance the endothelialisation of synthetic surfaces. We believe that the presence of endothelial stem cells combined with the new micropatterning technique are two conditions improving endothelialisation on a synthetic surface. A study with the use of endothelial stem cells has never been carried out and even less on a synthetic surface. We are convinced that determining the promoter signalling of the endogenous stem cells will allow future treatment in synthetic arterial prostheses.

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