Up-regulation of thrombomodulin is inducible on an endothelialized polyester

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The concept of endothelial cell seeding of vascular prostheses is designed as a method for improving long-term patency substitutes because endothelium is considered as the haemocompatible surface of reference. The assessment of the functionality of cells lining a biomaterial is thus of crucial importance. We have reported encouraging results concerning the ability of a polyester coated with albumin and chitosan (M 11) to be lined by a confluent monolayer of cultured human endothelial cells (EC). The aim of the present work was to study the expression of thrombomodulin (membrane glycoprotein responsible for anticoagulant activity) in EC lying on M.11 by anticoagulant activity and mRNA level with and without stimulation. Results obtained in basal conditions showed that EC on M.11 have a comparable expression of TM mRNA when compared to control (EC on tissue culture plates) despite a lower TM surface activity for EC on the biomaterial. In terms of ratio (stimulated cells to unstimulated ones) the response in activity for EC on M.11 is comparable to that of the control. These results indicate that cells lying on M.11 are able to respond to physiological-like stimuli, despite a tendency for these cells to express a procoagulant phenotype when compared with control EC.

1. Introduction

Among methods for improving long-term patency of vascular grafts, the concept of endothelial cell seeding on prostheses is designed to provide more performant substitutes because endothelium is considered as the haemo-compatible surface of reference. Indeed the endothelial cell surface is a dynamic system for the mechanisms inhibiting and promoting activation of the coagulation, the so-called haemostatic balance. One of the most important regulatory factors in the anticoagulant system is the thrombomodulin (TM) anchored on the endothelial cell membrane. This constitutively expressed glycoprotein is a specific receptor for thrombin and exhibits two complementary anticoagulant functions: one is to decrease the circulating level of generated thrombin which catalyses clot formation, and the other is to convert thrombin into a potent protein C activator, by formation of a 1:1 complex between TM and thrombin; this complex activates protein C into activated protein C which functions as an anticoagulant by inactivating activated factors V and VIII. Thus, blood coagulation at the endothelial cell surface is considered to be controlled by the changes of TM expression [1]. Several substances can modulate the expression of TM either by decreasing TM antigen and activity or by increasing them such as thrombin and dibutyryl-cyclic AMP (Bt_2cAMP). We have reported encouraging results concerning the ability of a polyester to be lined by a confluent monolayer of cultured human umbilical vein endothelial cells (HUVEC) [2]. The aim of the present study was to explore whether an endothelialized polyester could provide a non-thrombogenic surface able to function as the original endothelium, especially concerning TM regulation.

2. Materials and methods

2.1. Material

The knitted polyester was provided by the Bakoulev Cardiovascular Institute in Moscow as prostheses coated with albumin and sulfonated chitosan (hemisynthetic polysaccharide) (called M.11). It was cut and sterilized as previously described [2].

2.2. Cell culture and stimulation

HUVEC were harvested and cultured as previously described [2]. For experiments, cells from passage 1 or 2 were seeded on to tissue culture plates (TCP as

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control) or on to M.11 as previously described [2]. Seeding was performed at 4×10^4 HUVEC/cm^2 on the material and 2×10^4 HUVEC/cm^2 on plastic control, and experiments were carried out with 1–3 days post-confluent cultures. Some confluent monolayers of HUVEC were stimulated with 5 mM Bt_2cAMP (Sigma Chemical, USA) for 6 h at 37 °C for TM activity in Dulbecco's modified essential medium supplemented with 0.4% Ultroser G (Gibco, France) and with either 5 mM Bt_2cAMP or 1 U/ml of human thrombin (Diagnostica Stago, France) for various times for the determination of mRNA content. Other monolayers were left unstimulated.

2.3. Thrombomodulin surface activity

TM activity was measured by a functional assay according to Archipoff *et al.* [1] with some modifications. Briefly, confluent HUVEC were incubated with thrombin (0.1 U/ml) and protein C (65 nM) (Sigma) for 1 h at 37 °C. The reaction was blocked by addition of hirudin (2.5 a.t.u./ml) (Diagnostica Stago) for 5 min. The amidolytic activity of generated activated protein C (APC) was measured using substrate S 2366 (5 mM) (Biogenic, France). The concentration of APC was determined by reference to a standard curve established with known amounts of APC (Diagnostica Stago). Each point is the mean ± S.D. of triplicate measurements.

2.4. RNA isolation and enzymatic amplification

For total RNA preparation, 25 cm^2 confluent HUVEC on TCP or M.11 were trypsinized and total RNA were extracted with RNeasy Total RNA Kit (Qiagen s.a., France). For enzymatic RNA amplification of TM, 1–5 μg total extracted RNA were used for the Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) using primers corresponding to nucleotides 1071 to 1100 (5′ primer) and nucleotides 1262 to 1291 (3′ primer), according to Archipoff *et al.* [1] (Genset, France). β-actin mRNA was chosen as housekeeping gene and amplification was performed on the same total RNA extract.

3. Results and discussion

According to previous results [2], 5 days culture were necessary for seeded HUVEC on M.11 to produce a confluent monolayer comparable in terms of density to that on TCP. Enumeration after trypsinization showed 96 750 ± 42 386 HUVEC/cm^2 on TCP and 111 540 ± 43 707 HUVEC/cm^2 of M.11 estimated surface. Cells were characterized by their von Willebrand Factor secretion, as previously described [2]. The TM surface activity assessed by the chromogenic method showed that HUVEC on TCP expressed significantly more TM activity than HUVEC on M.11 during basal conditions, as well as after stimulation for 6 h with Bt_2cAMP (Fig. 1). However, the ratio of APC generated by stimulated cells to APC generated by unstimulated ones is comparable for HUVEC on TCP

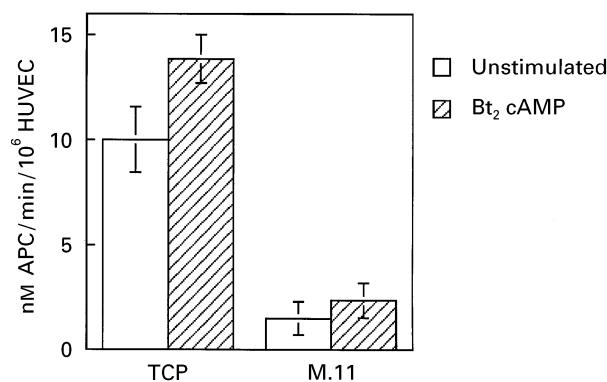

Figure 1 Results of TM activity on HUVEC lying on TCP and M.11, with or without stimulation, expressed as nM APC (activated protein C) per minute and 10^6 HUVEC.

(1.38) with HUVEC on M.11 (1.46). These latter ratios are consistent with those obtained by Ishii *et al.* [3] on HUVEC cultured and stimulated in conditions comparable to the present. In the same way, Archipoff *et al.* [1] reported a 28 ± 3% increase in TM activity for saphenous vein endothelial cells upon 10^{-3} M Bt_2cAMP stimulation for 4 h.

The level of mRNA for TM was assessed in HUVEC using the RT-PCR method. The amount of mRNA was normalized to that of the mRNA for β-actin, which is not affected by the different agents used in the present study. Resting HUVEC on TCP or M.11 showed constitutive expression of TM mRNA with no detectable difference, despite the difference observed with surface molecule activity. Stimulated cells on TCP (either by 5 mM Bt_2cAMP or 1 U/ml human thrombin) led to an increase of mRNA content for the first 2 h before it returned to basal expression, 4 h after incubation with stimulatory agents. These results are not in agreement with results presented by Bartha *et al.* [4] where TM mRNA level was augmented after 4 h in similar thrombin-activated conditions on human saphenous vein endothelial cells. This could be partially explained by three reasons: (i) cell origin, (ii) range of subcultures at the time of experiments and, finally, (iii) cell culture conditions which are of major importance [1].

Stimulated HUVEC on M.11 seem to produce an identical pattern in mRNA expression with an increase around 2 h stimulation. However, further studies are necessary to quantify mRNA content of HUVEC seeded on these two supports and to measure the extent of thrombin and Bt_2cAMP stimulation. For this purpose, we are elaborating a probe for TM mRNA Northern blot analysis.

4. Conclusion

The assessment of the functionality of cells lining a biomaterial is of crucial importance in order to build a hybrid artificial vascular substitute made of endothelial cells and vascular graft. The latter concept is known to improve long-term patency [5]. The present work indicates that the chitosan- and albumin-coated polyester tested in our laboratory can provide a good

support for endothelial seeding, because the colonized cells are able to respond to physiological-like stimuli, despite a tendency for these cells to express a procoagulant phenotype [2]. Thus, the physiological haemostatic balance on this polyester could be modified, as shown by the present results and those reported previously [2].

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