In vitro assessment of endothelial cell adhesion mechanism on vascular patches

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Endothelial cell (EC) seeding of small caliber vascular grafts prior to their implantation has proved to significantly improve long-term patency in humans. We have previously demonstrated that a monolayer of EC could be obtained on type I collagen-coated knitted ultrathin polyster grafts (InterVascular, La Ciotat, France). Thus, the aim of the present work was to understand the nature of cell adhesion mechanisms involved in the cell /biomaterial interface, using HemaCarotid® (InterVascular) patches made of type I collagen-coated knitted ultrathin polyster (type I collagen is used to coat patches to attain low permeability). By means of quantitative attachment tests, adhesion blocking assays, RT–PCR for the expression of β_1 integrin mRNA, indirect immunofluorescence with antivinculin antibody, we were able to show that EC are able to adhere to such surfaces by the means (non-unique) of cell surface receptors of the β_1 integrin group. However, the latter are probably down-regulated at the cell/biomaterial interface.

1. Introduction

It is well known that the replacement of arteries with purely synthetic vascular prostheses often leads to the failure of such reconstructions when small-diameter or low-flow locations are concerned, due in part to the thrombogenicity of the internal graft surface. In order to improve long-term patency of these grafts, the concept of endothelial cell (EC) seeding has been suggested because this metabolically active endothelial surface plays a major role in preventing *in vivo* blood thrombosis and because vascular grafts placed in humans do not or rarely [1–3] spontaneously form an endothelial monolayer, whereas they do in animal models. To date, the *in vitro* complete and preformed endothelial lining at the time of implantation demonstrated its superiority in terms of significantly increased patency of the grafts that underwent endothelialization several years earlier [4]. We have previously reported encouraging results about retention of ECs subjected to fluid shear stress [5] when cells were lining a collagen coated knitted ultrathin polyester graft (InterVascular, La Ciotat, France).

As far as EC adhesion is involved in cell/biomaterial interaction and because the prosthesis is impregnated with type I collagen to attain low permeability, the aim of the present study was to understand the nature of cell adhesion mechanism under static conditions and to investigate whether the material could influence this adhesion, using HemaCarotid® (InterVascular) patches made of collagen-coated knitted ultrathin polyester. Collagen, a component of the extracellular matrix, contains biologically active sites such as Arg-Gly-Asp (RGD) and Asp-Gly-Glu-Ala (DGEA) sites [6] known to interact with and to bind to integrins present on the cell surface. More precisely, β_1 integrin subunits, expressed on ECs, attach to adhesive sites and anchor the cytoskeleton to the plasma membrane. Integrins thus mediate bidirectional information transfer [7] which is transduced across the plasma membrane into a variety of signaling transduction pathways.

2. Materials and methods

2.1. Endothelial cell culture

HUVEC (human umbilical vein endothelial cells) were isolated and cultured as previously described [8] according to the modified original method of Jaffe *et al.* [9]. At confluency, cells were harvested for experiments by trypsinization, and HUVEC were seeded either in 96-well microtitre plates or Petri dishes from Nunc (Roskilde, Denmark). Experiments were carried out with cultures at passage 1 or 2.

2.2. Biomaterial under test

In order to miniaturize areas of interest, circular pieces were stamped from patches (HemaCarotid®) to fit the bottom of 96-well plates. In order to avoid cell adhesion to the plastic of wells, which could occur through material meshes during seeding, an agarose layer was prepared and poured into the well as previously described [10].

2.3. Attachment tests

HUVEC were seeded on the inner surface of patches and controls at three different densities (15 or 60 or $150 \times 10^3\,cm^{-2}$) in serum-free medium and for three different times (three to seven culture wells per series and time): 1 h, 3 h, 6 h. At the end of the incubation period, quantitative attachment tests were performed as previously described [11] and compared with controls, i.e. culture plates coated with the same collagen as that used to coat patches.

2.4. Adhesion blocking assays

In order to confirm the involvement of β_1 integrin subunit in HUVEC adhesion to patches, experiments were undertaken with a monoclonal antibody (mAb) against integrin β_1. Briefly, HUVEC ($5 \times 10^5\,ml^{-1}$) was incubated in suspension for 10 min at 37 °C with $10\,\mu g\,ml^{-1}$ of anti-integrin β_1 (Gibco BRL) followed by seeding ($150 \times 10^3/cm^2$) on patches and coated culture plates ($n = 4$ per series). One hour later, the attachment measuring procedure was performed (see above). Controls were performed using a non-specific antibody (mAb against RNA polymerase). At least two experiments were performed.

2.5. Reverse transcriptase–polymerase chain reaction (RT–PCR)

First strand cDNA was synthesized by RT of 3 μg total RNA isolated from HUVEC using RNeasy total RNA kit (Qiagen) and amplified by Taq DNA polymerase dissolved in PCR buffer (Gibco, BRL) in a 50 μl reaction containing 0.2 nM and 1.2 nM of β_1 primers (sense: 5′CGGGGTGAATGGAACAGGGG3′ antisens: 5′CACACTCGCAGATGCCCCGGCC 3′) (GENSET Oligos). The PCR profile consisted of 5 min of denaturing at 94 °C, followed by 30 cycles of 1 min of denaturing at 94 °C, 30 s of annealing at 63 °C, 1 min of extension at 72 °C, and a final extension step of 10 min. The PCR product (15 μl) was separated by a 1% agarose gel and stained with ethidium bromide to identify a 600 bp product. mRNA expression of β-actin was used as positive control.

2.6. Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed at day 3 after cell seeding on the following substrates: collagen-coated culture plates and HemaCarotid patches. The medium was removed and cells fixed with 4% paraformaldehyde. Thereafter, specimens were incubated with antivinculin primary antibody (Sigma) for 2 h at 37 °C, washed before incubation with secondary antibody Alexa – labeled goat antimouse immunoglobulin purchased from Molecular Probes. Stained specimens were photographed.

2.7. Statistical analysis

The *U*-Mann–Whitney non-parametric test was used for statistical analysis. $p < 0.05$ was considered as significant.

3. Results

The attachment kinetics of HUVEC on patches are presented in Fig. 1a. Previous experiments have established that EC enumeration could be quantified by absorbance measurement with a direct evident relationship between the number of cells and *N*-acetyl-β-D-hexosaminidase (data non shown). Cells did not adhere on agarose layer (data not shown). A maximum of seeded cells is reached within 1 h for a given cell density. For a given incubation time, the absolute number of attached cells on patches increases as the seeded cell number increases: 6660 ± 1575, $19\,200 \pm 3600$, $44\,550 \pm 16\,650$ for 15×10^3, 60×10^3, 150×10^3 seeded cells cm^{-2}, respectively at 1 h. When comparing the evolution of cell number over time and for a given seeding density, it appears that there is a significant cell loss for the two highest densities and between 1 and 6 h ($p < 0.02$).

From Fig. 1b, it appears that cell–substrata adhesion was partially blocked by the mAb against β_1 integrin in a comparable extent: -46% on coated culture plates and -41% on HemaCarotid patches. Nevertheless the inhibitions were significant compared with their matched controls performed with non-specific mAb ($p = 0.02$).

Fig. 2 shows vinculin containing focal contacts clustered in cellular extensions in an apparent attempt

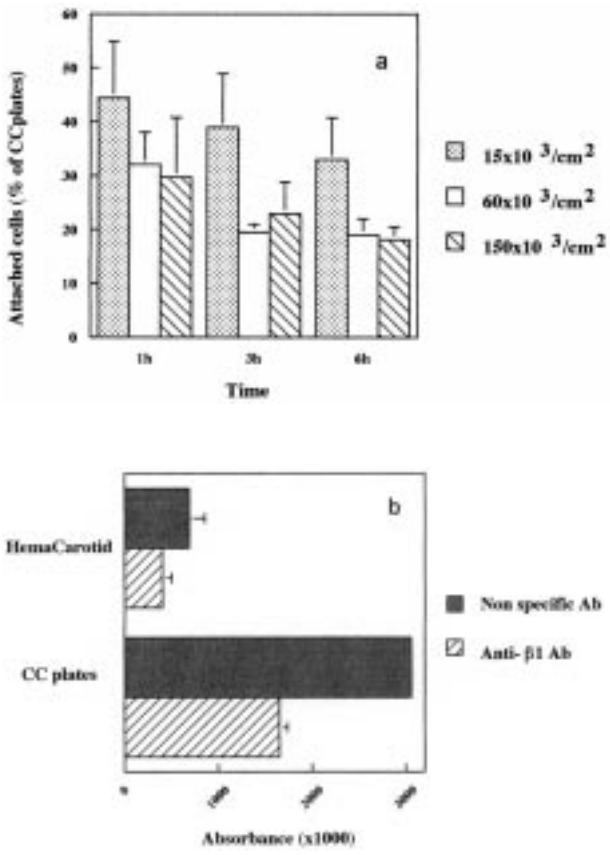

Figure 1 Histograms of EC adhesion (a) and (b) of adhesion blocking assays on HemaCarotid® patches in comparison with type I collagen coated culture (CC) plates.

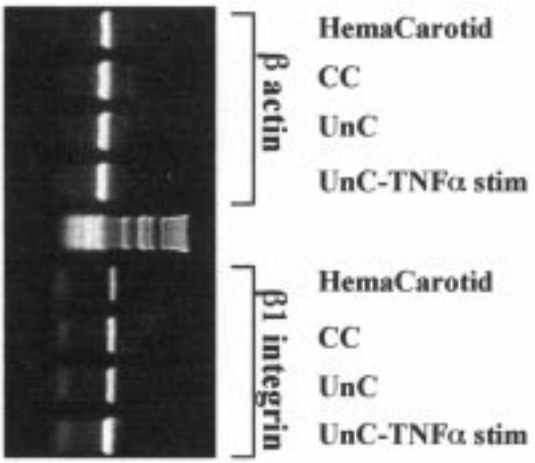

Figure 2 Reverse transcriptase-PCR showing the β_1 mRNA level of HUVEC in contact with: HemaCarotid, uncoated culture plates (UnC), coated culture plates (CC): of HUVEC stimulated by tumor necrosis factor-α (TNF-α) (20 ng ml^{-1} 18 h) on uncoated culture plates (UnC-TNF-αstim). β-actin is used as control of mRNA expression.

to spread their cell bodies over both substrates. Focal contacts are visible as streak-like deposits predominantly at the periphery, as expected. RT–PCR of total RNA extracted from HUVEC in contact or not with patches for 6 h demonstrates the presence of β_1 integrin-mRNA whatever the substrate (Fig. 3). Nevertheless, the β_1 mRNA level of HUVEC in contact with patches is expressed in a lower extent compared with coated or uncoated culture plates.

4. Discussion

A major challenge for recent vascular surgery is to produce functional prosthetic grafts for use in patients. Besides exciting experimental approaches consisting of developing completely biological tissue-engineered human blood vessels [12, 13], reported clinical trials with endothelialized prosthetic vascular grafts demonstrated the validity of such an approach to overcome the

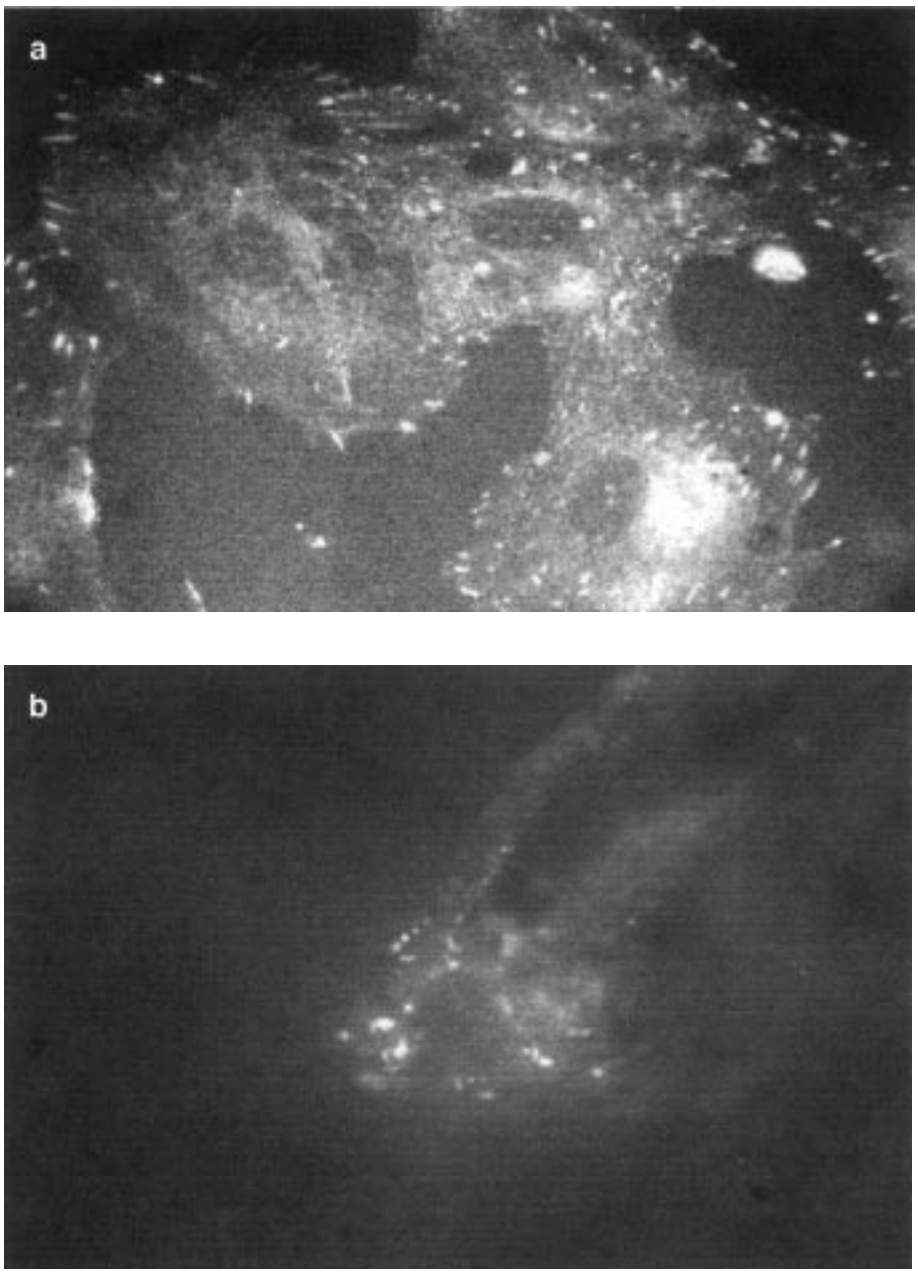

Figure 3 Localization of vinculin at day 3 by indirect immunofluorescence microscopy (original magnification × 400) in HUVEC seeded on (a) coated culture plates and (b) on patches.

problem of low patency rate for arterial bypass below the knee [4].

Successful cell adhesion and spreading on artificial surfaces are prerequisites for cell colonization. Protein precoating of implant surfaces may influence the biocompatibility of implant materials so as to support cell coverage. In this respect cell culture models offer the possibility of evaluating different effects of biomaterials on a variety of cellular responses. Thus, we focused our interest on the adhesion process of ECs on a polyester precoated with type I collagen. Cell adhesion is a complex process depending on multiple interactions between surface topography, matrix receptors such as integrins and diverse cytoplasmic proteins [14–16]. Integrins not only play a critical role in cell anchorage to their substrates but also in other numerous biological processes that include cell migration, cell growth and cell differentiation.

Several stages of EC adhesion to substrates can be distinguished. The initial attachment is followed by the formation of focal adhesion sites and subsequent cell spreading [17].

Whatever the cell seeding density, a maximal endothelial attachment was achieved after patches had been incubated for 1 h with cultured ECs suspended in serum-free medium. When cells were maintained in serum-free medium for longer than 1 h, it resulted in a 40% loss by 6 h.

These results are consistent with those of Anderson *et al*. [18]. However, the results of our plating efficiency at 1 h seem lower than those reported [18]. These authors established that cell attachment was directly related to the number of cells available in the cell suspension medium, with a saturation cell density of about $3.5 \times 10^5\ cm^{-2}$ in the case of polytetra-fluoro-ethylene (PTFE) grafts precoated with type I collagen. Thus the maximum number of cells capable of attaching to patches, despite other substrates as ours, was probably not reached in our experiments. Our data indicate that cell–substratum adhesion occurred via β_1 integrin but other integrins are probably involved and have to be explored. The present work focused on β_1 integrin: (1) because it is the predominant β isoform expressed in HUVEC [19] and (2) because at least $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$ all bind to collagen [20].

Focal adhesion contacts, which are known to be formed at the sites of linkage of the cytoplasmic tails of integrins with cytoskeletal proteins, involve a number of signaling proteins, such as focal adhesion kinase, paxillin, α-actinin and tensin.

Vinculin, a 130-kDa cytoskeletal protein localized in focal contacts, could be visualized in cells lying on the coated artificial surface.

Thus, as cell adhesion, a prerequisite for subsequent EC proliferation and coverage, is observed on patches from our study, the nature of signal transduction by integrin-mediated adhesion as well as the signaling events activated by shear stress remain to be explored.

Acknowledgments

This work was supported by grants from the Fondation de l'Avenir, Pôle Aquitain de Génie Biologique et Médical. InterVascular is gratefully acknowledged for supplying HemaCarotid patches.

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Received 12 May
and accepted 2 June 1999