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Capability of human umbilical cord blood progenitor-derived endothelial cells to form an efficient lining on a polyester vascular graft in vitro

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Abstract

One of the goals of vascular tissue engineering is to create functional conduits for small-diameter bypass grafting. The present biocompatibility study was undertaken to check the ability of cord blood progenitor-derived endothelial cells (PDECs) to take the place of endothelial cells in vascular tissue engineering. After isolation, culture and characterization of endothelial progenitor cells, the following parameters were explored, with a commercial knitted polyester prosthesis (Polymaille® C, Laboratoires Pérouse, France) impregnated with collagen: cell adhesion and proliferation, colonization, cell retention on exposure to flow, and the ability of PDECs to be regulated by arterial shear stress via mRNA levels. PDECs were able to adhere to commercial collagen-coated vascular grafts in serum-free conditions, and were maintained but did not proliferate when seeded at 2.0×10^5 cm^{-2}. Cellularized conduits were analyzed by histology and histochemical staining, demonstrating collagen impregnation and the endothelial characteristics of the colonizing cells. Thirty-six hours after cell seeding the grafts were maintained for 6 h of either static conditions (controls) or application of pulsatile laminar shear stress, which restored the integrity of the monolayer. Finally, quantitative real-time RT-PCR analysis performed at 4 and 8 h from cells lining grafts showed that MMP1 mRNA only was increased at 4 h whereas vWF, VE-cadherin and KDR were not significantly modified at 4 and 8 h. Our results show that human cord blood PDECs are capable of forming an efficient lining and to withstand shear stress.

Keywords: Progenitor-derived endothelial cells; Biocompatibility evaluation; Vascular grafts; Shear stress; Gene regulation

1. Introduction

The isolation of endothelial progenitor cells (EPCs) from human peripheral and umbilical cord blood [1] has generated great hope in the fields of cellular therapies and regenerative medicine, as these precursors have been demonstrated to be of particular benefit in animal models, as well as in some clinical studies, after injection into the circulatory system or at the site of injury in case of ischemic vascular diseases.

The outlook for EPC-based therapy for cardiovascular disease tissue engineering as well as for cancer has been the subject of recent reviews [2–7]. If EPCs directly injected are able to differentiate in situ, they could also differentiate in vitro, and be proposed for applications in the biomedical field. Indeed, despite numerous attempts all over the world through different approaches over a number of decades [8,9], the engineering of a small-caliber blood vessel substitute for use in peripheral and coronary bypass surgery is still a challenge. As for a normal blood vessel, non-thrombogenicity is provided by the vascular endothelium; thus, to

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construct blood vessels by tissue engineering, one technique used is the seeding of endothelial cells (ECs). At present, EC seeding of synthetic prostheses can be performed, in humans, with autologous EC, requiring the harvesting of cells, followed by culturing and amplification for several weeks and then seeding onto coated grafts [10–12]. As large amounts of seeded ECs are needed, a reliable source of these cells is a major concern in vessel tissue engineering.

Isolating EPCs are relatively easy, and their capacity to expand in culture while retaining their ability to switch into ECs has led to their application in developing constructs. In line with this, they have been used for the endothelialization of vascular artificial stents [13], human pulmonary valves [14] and prostheses [15–17] instead of mature endothelial cells. Furthermore, the first clinical trials have been reported using EPCs antibody-coated coronary stents [18,19]. In the present work our overall hypothesis was that umbilical cord blood progenitor-derived ECs (PDECs) could represent a reliable cell source for seeding, with the following benefits and for the following reasons: the umbilical cord contains a richer source of stem cells that can easily be extracted and cryopreserved [20] than peripheral blood or bone marrow [21]. It provides immediately available cells, with no risk to the donor and a low risk of transmitting infectious diseases [22]. These cells have a higher proliferation rate and superior colony-forming ability than peripheral blood-derived EPCs [23], and possess a superior in vivo potential to form normal functional long-lasting vessels in vivo, as recently demonstrated in SCID mice [24].

In vivo, ECs are continuously exposed to mechanical (tangential fluid shear stress, cyclic circumferential strain and blood pressure) and biochemical (VEGF, IL-1, TNFα, etc.) stimuli, which are important modulators of vascular cell functions, growth and structure at both the protein and mRNA levels. The mechanisms by which ECs sense mechanical stimuli and convert them into biochemical signals have been partly elucidated [25,26]. In particular, vascular endothelial growth factor receptor 2 (VEGFR2, Flk1/KDR) and vascular endothelial cadherin (VE-cadherin) of the adherens junction were shown to act as shear stress cotransducers and belong to a mechanosensory complex [27].

Thus, the capability of umbilical cord blood-derived EPCs to form an endothelial cell lining on a commercial vascular prosthesis in vitro, and their ability to withstand the shear stress of the blood flow and to be responsive to shear stress at mRNA levels were the subjects of investigation in the present study.

2. Materials and methods

2.1. Isolation, culture and characterization of PDECs

Human umbilical cord blood samples were collected from donors in accordance with the French legislation. The samples were immediately processed in the laboratory for isolation of EPCs according to the procedure described by Bompais et al. [28]. PDECs were characterized as endothelial by immunofluorescent stainings for von Willebrand Factor (vWF), KDR, CD31 and incorporation of Dil-Ac-LDL, and by FACS analysis for VE-cadherin, as in our previous papers [29,30].

2.2. Vascular graft under test

The vascular graft POLYMAILLE® C is a knitted polyester prosthesis, kindly provided by Laboratoires Pérouse (France), impregnated with collagens I + III of bovine origin that was sterile at the time of use.

2.3. Cytotoxicity, cell attachment and proliferation assessments

Cytotoxicity assessment was performed according to standard ISO/EN 10993 part 5 guidelines using PDECs plated in 96-well plates (seeding density: 2.0×10^5 cm^{-2}) and grown until confluency, obtained 4 days after seeding. Briefly, cell monolayers were incubated for 24 h at 37 °C (six wells per series and concentration) with the original extract (i.e. pure undiluted) or to a dilution series of the original extract. At the end of the incubation period, the material or control extracts were removed and two quantitative colorimetric tests were performed: the cell viability (Neutral Red assay) and the cell metabolic (tetrazolium-based) activity (MTT assay).

For cell attachment measurement, circular pieces were stamped from prostheses to fit the bottom of 48-well plates. In order to avoid cell adhesion to the plastic of wells, which could occur during seeding, an agarose layer was prepared and poured into the wells as previously described [31]. Cells were seeded on the inner surface of patches and controls (culture plates coated with gelatin (0.2% (w/v)) at two different densities (0.2 or 2.0×10^5 cm^{-2}, corresponding to cell seeding density (CSD) 1 and CSD2, respectively) in serum-free medium (M199, GIBCO®) for 1, 3 and 24 h ($n = 8$). At the end of the incubation period, quantitative attachment tests were performed as previously described [32] and compared with the controls.

Cell proliferation was assessed on circular pieces of prostheses fitted the bottom of 24-well plates previously filled with an agarose layer as above and compared with gelatin-coated control wells. Cells were seeded at two different densities (0.2 or 2.0×10^5 cm^{-2}) in ECGM-MV2 medium PromoCell® and cultured for 1, 2, 4, 7 and 9 days ($n = 6$) with medium changes every 3 days. At the end of the incubation period, cell proliferation was evaluated with the MTT test because of a satisfactory correlation between cell numbers and absorbance [33].

2.4. Grafts endothelialization and flow experiments

The POLYMAILLE® C graft (6 mm internal diameter and 20–25 cm length) was filled with the PDEC suspension: 2×10^6 cells ml^{-1} of ECGM-MV2 medium representing a

seeding cell density of 2.0×10^5 cells cm^{-2}. After end graft ligature, the PDEC seeded graft was placed in a rotative device (Endostrabilisator, Biegler, Mauerbach, Austria) for 4 h in a 5% CO_2 incubator to obtain homogeneous coverage. After seeding, the ligatures were removed, the endothelialized grafts were cut into two equal pieces of 12 cm length and then maintained for 36 h in the incubator before being submitted to static and flow conditions for 4 and 8 h ($n = 3$). The flow circuit was a closed system consisting of flexible plastic tubes, a two roller-pump which delivered a laminar pulsatile flow. An ultrasound transducer probe (Flowmeter T110, Transonic Systems Inc., NY, USA) was used to register flow. The shear stress, calculated with Hagen–Poiseuille's law, averaged 15 dyn cm^{-2}, which is comparable to the physiological range in a human major artery and known to induce the expression of several immediate early genes in vitro. A pressure of 120/60 mm Hg and a temperature of 37 °C were maintained throughout the experiments. The prostheses were exposed to ECGM-MV2 medium supplemented with 8% low-molecular-weight Dextran (mol. wt. 75,000 Da; Sigma–Aldrich) to obtain a viscosity of the solution of 0.04 poise. Dextran was proven to be non-cytotoxic at that concentration (data not shown).

2.5. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

After exposure to 4 and 8 h of flow, cells were detached with trypsin–EDTA solution (0.125% trypsin and 12.5 mM EDTA). The cells were rinsed in cold phosphate-buffered saline (PBS), then total RNA was extracted and treated according to Ref. [40]. Table 1 shows the different primers used with the temperature and the length of PCR product. Data were analyzed with the iCycler IQ™ software, and compared by the ΔΔCt method. Each PCR was performed in duplicate, and in at least triplicate for PCR yield validation. All experiments were performed at least three times, and data are expressed, from the ΔΔCt method, as mean of relative quantity of mRNA, and as upper and lower values. In this method, the normalized mRNA level in ECs under static conditions was arbitrarily set at "1". The statistical significance of differences in mRNA expression between static and dynamic conditions for each coating was analyzed by the Relative Expression Software Tool (REST©) for groupwise comparison and statistical analysis of relative expression results in real-time PCR [34].

2.6. Morphological examination

Histological analysis was performed on cellularized grafts both in the static condition and after 6 h of shear stress. After fixation in formalin, grafts were classically embedded in paraffin, cut transversely into 7 μm sections, and stained with hematoxylin and eosin (H&E). Masson's trichrome staining was used to visualize collagen and immunohistochemistry was performed with immunoperoxidase-labeled antibodies against CD31 and CD34. Stained specimens were photographed. Moreover, additional grafts fixed in formalin were washed twice with PBS and the cells permeabilized in 0.1% (w/v) Triton X-100 in PBS for 1 min. The samples were then washed briefly in deionized water and incubated for 15 min at room temperature with 0.5 μm Sytox green nucleic acid stain (Molecular Probes) made up in water. Following incubation, the samples were washed in PBS before being analyzed under a microscope. Samples were viewed using a Leica confocal microscope at 10× magnification which was specifically tuned to detect the Sytox stain and to minimize the background or autofluorescence. Images were captured as a *z*-series of 30 images with a *z*-step of 8–10 μm. These were then maximally projected to produce a single image. Finally, a quantitative analysis of fluorescence intensity was performed. The initial RGB-coloured image was decomposed into R (red), G (green) and B (blue) components. Each of them was encoded with 256 gray levels. The green intensity distribution of the initial image was evaluated as the gray level distribution of the G component image. The mean distribution evaluated over five different samples was exploited as a quantitative tool for comparing "static" and "shear stress" experiment conditions.

Table 1
List of the different primer pair sequences that were used for quantitative real-time RT-PCR with the forward and reverse sequences, the optimal temperature and the length of the PCR products.

Oligonucleotides	Sequences	Temperature T_m (°C)	Length of PCR product (bp)
vWF	Sense (5'-'3): TGGAGCAGCAAAGGGACGAGA	59.5	66
	Antisense (5'-'3):TAGGAGGAGGGGCTTCAGGGG	60.2	
MMP1	Sense (5'-'3): GCTGGGAGCAAACACATCTGA	55.1	89
	Antisense (5'-'3): GGCTTTCTCAATGGCATGGTC	55.9	
VE-cadherin	Sense (5'-'3): GGCTCAGACATCCACATAACC	51.0	145
	Antisense (5'-'3): CTTACCAGGGCGTTCAGGGAC	57.3	
KDR	Sense (5'-'3): AAGTGGAGGCATTTTTCATAA	48.6	228
	Antisense (5'-'3): CATAAGGCAGTCGTTCACAAT	49.5	
Po	Sense (5'-'3): ATGCCCAGGGAAGACAGGGC	59.8	166
	Antisense (5'-'3): CCATCAGCACCACAGCCTTC	55.3	

VWF, von Willebrand factor; MMP1, MatrixMetalloProteinase 1; VE-cadherin, vascular endothelial-cadherin; KDR, VEGFR-2; Po is a housekeeping gene, chosen as the reference gene that encodes for a ribosomal protein and is not influenced by the experimental conditions.

2.7. Statistical analysis

Concerning cytotoxicity, cell attachment and proliferation, data are expressed as absorbance ratios. The statistical significance of differences between static and dynamic conditions was analyzed. The Mann–Whitney non-parametric *U*-test was used for statistical analysis. A p value of <0.05 was considered as significant.

3. Results

3.1. PDEC ability to colonize vascular prosthesis

Data obtained following incubation of PDECs with material extracts (Fig. 1a) during 24 h were expressed for Neutral Red and MTT assays as a percentage of the absorbance values obtained from cells incubated with negative control extracts (M199 culture medium without material). Phenol solution (64 g l^{-1}), used as a positive control, induced a high cytotoxic effect, as expected: cell metabolic activity and viability were less than 10% of that of the negative control. Extractables from grafts reduced significantly the cell metabolic activity of the original extract (100% v/v) as well as of the 50 vol.% material extract dilution, whereas this effect was abolished for the higher dilutions. No significant variation in cell viability in the presence of material extract was recorded. Thus, for the following experiments the prostheses and their fragments were extensively washed with M199 medium before cell seeding.

Concerning the attachment kinetics of PDECs on POLYMAILLE® C graft, previous experiments have established that endothelial cell enumeration could be quantified by the measurement of absorbance, with a relationship between the number of cells and *N*-acetyl-β-D-hexosaminidase being clearly evident (data not shown). Cells did not adhere to the agarose layer (data not shown). PDECs were able to adhere to commercial collagen-coated vascular grafts in serum-free conditions with absorbances decreasing for CSD1 (71.6 ± 9.7, 62.3 ± 4.7 and $53 \pm 7.3\%$ at 1, 3 and 24 h, respectively) or increasing for CSD2 (19.2 ± 5.4, 21.1 ± 5.2 and $28.8 \pm 4.1\%$ at 1, 3 and 24 h, respectively). The change in cell proliferation over time in the presence of complete culture medium was also monitored by a colorimetric assay (Fig. 1b and c): on control substrata PDECs either grew with a doubling time of approximately 40 h, consistent with Ref. [17], or main-

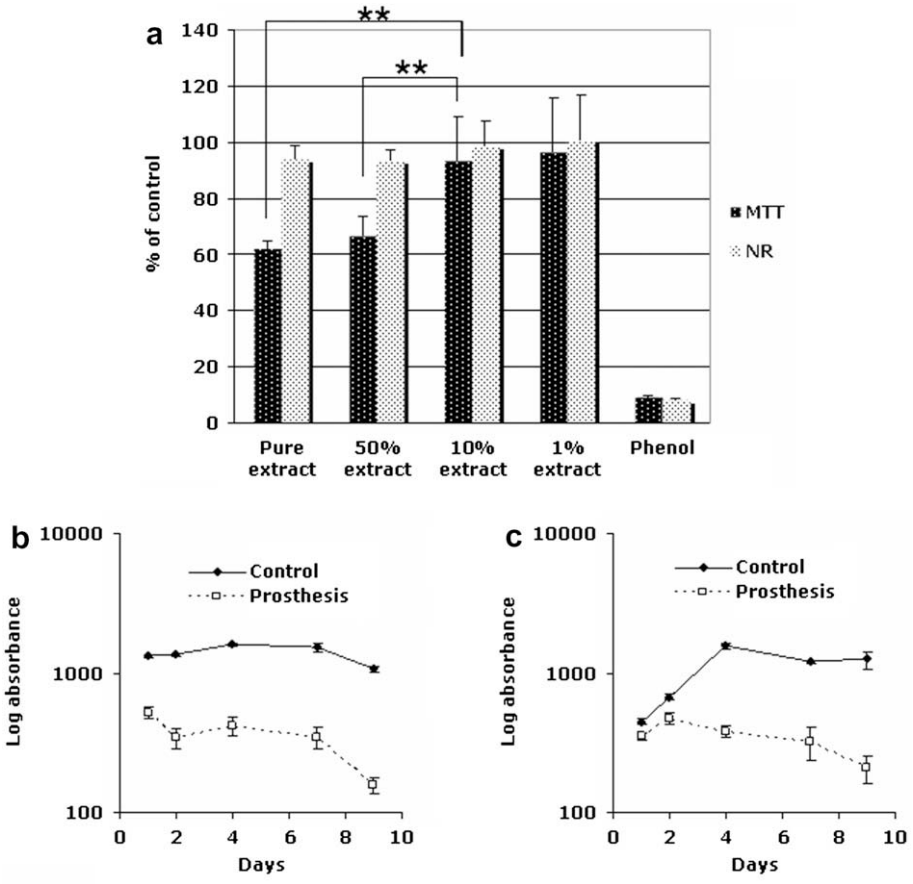

Fig. 1. Cytotoxicity and proliferation assessments of PDECs on vascular graft POLYMAILLE® C. (a) Results of cell viability (Neutral Red assay) and cell metabolic activity (MTT assay) colorimetric tests performed according to standard ISO/EN 10993 part 5 after incubation of monolayers with the original extract (i.e. pure undiluted) or a dilution series of the extract material. Data are expressed as absorbance ratios to control extracts. $^{**}p < 0.01$. (c,b) Cell proliferation after seeding at two different densities (0.2 and 2.0×10^5 cm^{-2}, respectively). At the end of the incubation period, cell proliferation was evaluated with the MTT test. Values are means ± SD.

tained their metabolic activity for the lowest or highest cell density, respectively. The plateau was either reached by day 4 (CSD1) or obtained immediately, corresponding to the confluency for CSD2 on controls. On the same timescale, cell proliferation did not occur on grafts whatever the cell seeding density; absorbance decreased for PDECs over time from day 2 for the lowest CSD (Fig. 1c), whereas it was maintained for longer at a higher CSD (Fig. 1b). For the further flow experiments the highest cell seeding density was chosen and samples were always submitted to shear stress 36 h after seeding.

3.2. PDEC retention on a shear-stressed colonized vascular prosthesis

All experiments were performed with each shear-stressed sample having a matched control maintained in static conditions. Analysis of H&E-stained sections of cellularized grafts seeded 36 h earlier then maintained for 6 h of either static conditions (Fig. 2A, C, E and G) or under shear stress (Fig. 2B, D, F and H) confirmed the presence of cells at the graft's luminal surface. Masson Trichrome staining clearly shows collagen impregnation of the pros-

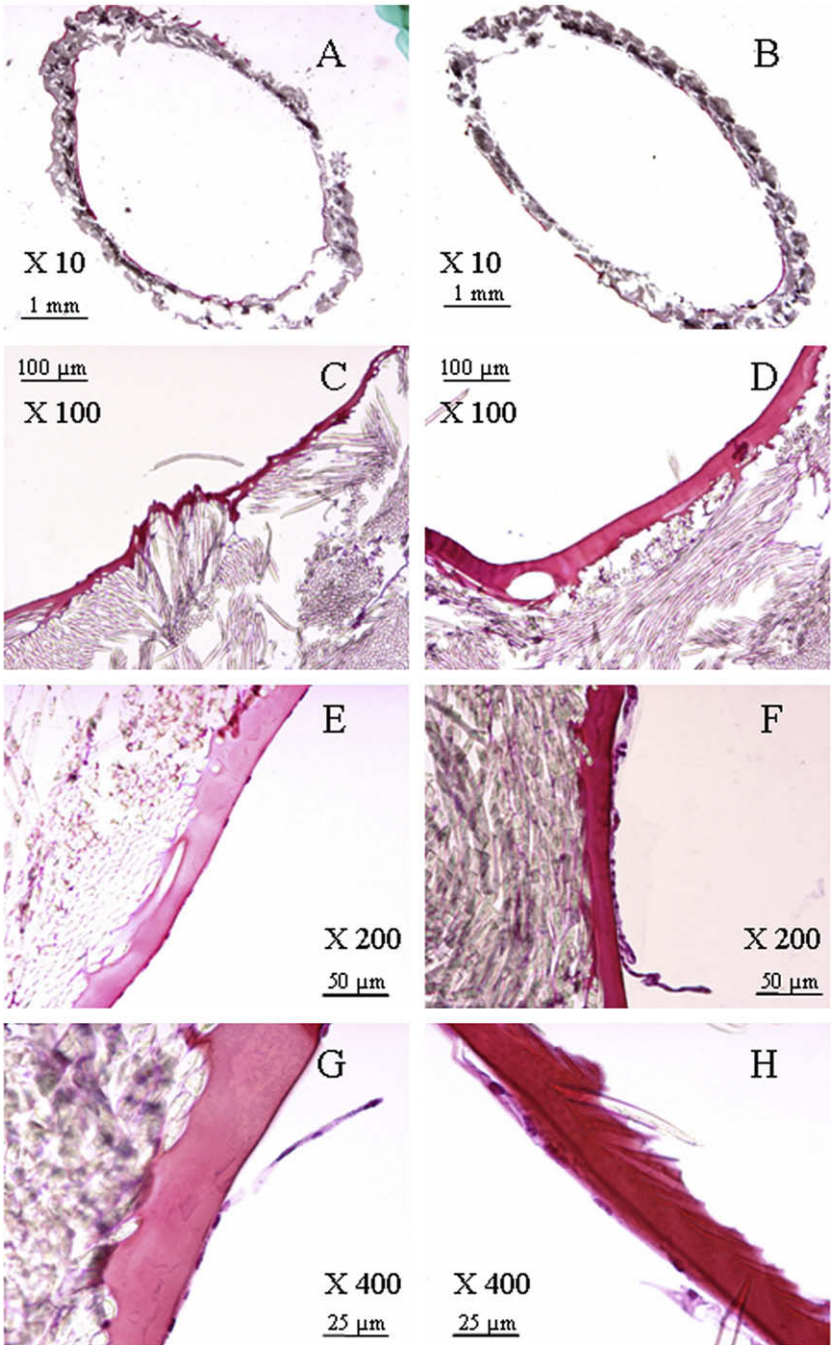

Fig. 2. Histology of endothelialized prostheses (HES) seeded 36 h earlier, then maintained for 6 h of either static conditions (A, C, E and G) or application of shear stress (B, D, F and H).

thesis (Fig. 3A and B), and immunohistochemical staining for CD34 and CD31 (Fig. 3C and D) demonstrates the endothelial feature of colonizing cells at the interface with collagen. Confocal laser scanning microscopy and the subsequent quantitative analysis of fluorescence are shown in Fig. 4. It seems that the integrity of the PDEC monolayer before its connection of cellularized prostheses in the flow circuit (Fig. 4A and B) is disrupted after 6 h of static conditions (Fig. 4C and D) but restored and enhanced after 6 h of shear stress (Fig. 4E and F). This is corroborated by the image processing, which shows a displacement between the fluorescence intensity analyzed along the entire length of the prosthesis between the static and flow conditions (Fig. 4G).

3.3. Gene expression in PDECs lining vascular prostheses in response to shear stress

Table 2 reports, as ratios, the relative expression of different mRNAs by PDECs seeded on grafts 36 h earlier then submitted to pulsatile laminar shear stress for 4 and 8 h. Expression of MatrixMetalloProteinase 1 (MMP1) mRNA in PDECs exposed to shear was significantly increased at 4 h, whereas in the same time, the expression of vWF, VE-cadherin and KDR was not significantly modified comparing flow to static conditions. Eight hours of shear exposure did not significantly enhance mRNA expression.

4. Discussion

One of the goals of vascular tissue engineering is to create functional conduits for small-diameter bypass grafting. Besides reported clinical trials with endothelialized prosthetic vascular grafts that demonstrated their validity [10–12], one can report several models developed to fabricate cellular vascular grafts by using cells alone, matrix alone or the combination of both [9]. When looking at the potential of EPCs for the fabrication of vascular grafts, the earliest study was reported in 2001 [16], while others proposed EPCs derived from umbilical cord blood [35,36]. Progress in biocompatibility and tissue engineering would today be inconceivable without the aid of in vitro techniques [37]. Our laboratory has been involved for many years in the development of a hybrid vascular substitute [38–41]. The present biocompatibility study was undertaken to check the ability of cord blood PDECs to take the place of endothelial cells in vascular tissue engineering. The following key parameters were explored to encompass biocompatibility evaluation: cell adhesion, a prerequisite for cell proliferation and/or cell colonization, followed by the evaluation of cell function at the cell/biomaterial interface. A critical first step is represented by assessment of the cytotoxicity of material extracts on cell cultures. Such a stage avoids direct contact between cells and materials, and evaluates the effect of possible contaminants that readily could be extracted from the biomaterials.

The cell culture response upon exposure to extracts was examined using two complementary assay methods, the biological and biochemical bases of which are quite different: the Neutral Red assay has been proposed for routine analysis of cell viability by many committees and organizations; and the MTT assay, for assessing the potential of novel antitumor agents [42], has been used for evaluating biocompatibility in vitro because of its reliability and sen-

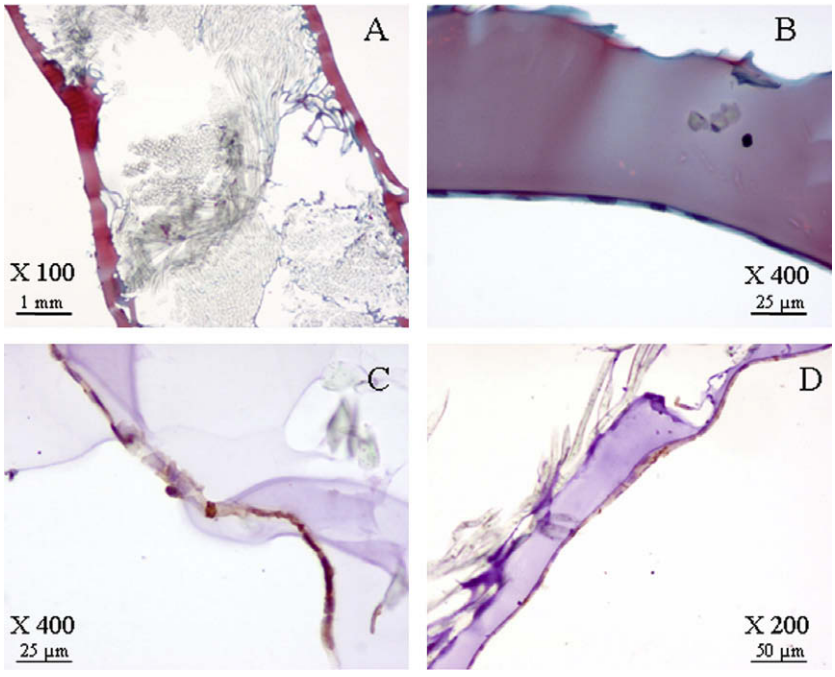

Fig. 3. Histochemical and immunohistochemical results of endothelialized prostheses. Masson's trichrome (A,B) staining showing collagen impregnation and cell monolayer. Immunohistochemical staining for CD34 and CD31 (C and D, respectively) showing endothelial cells.

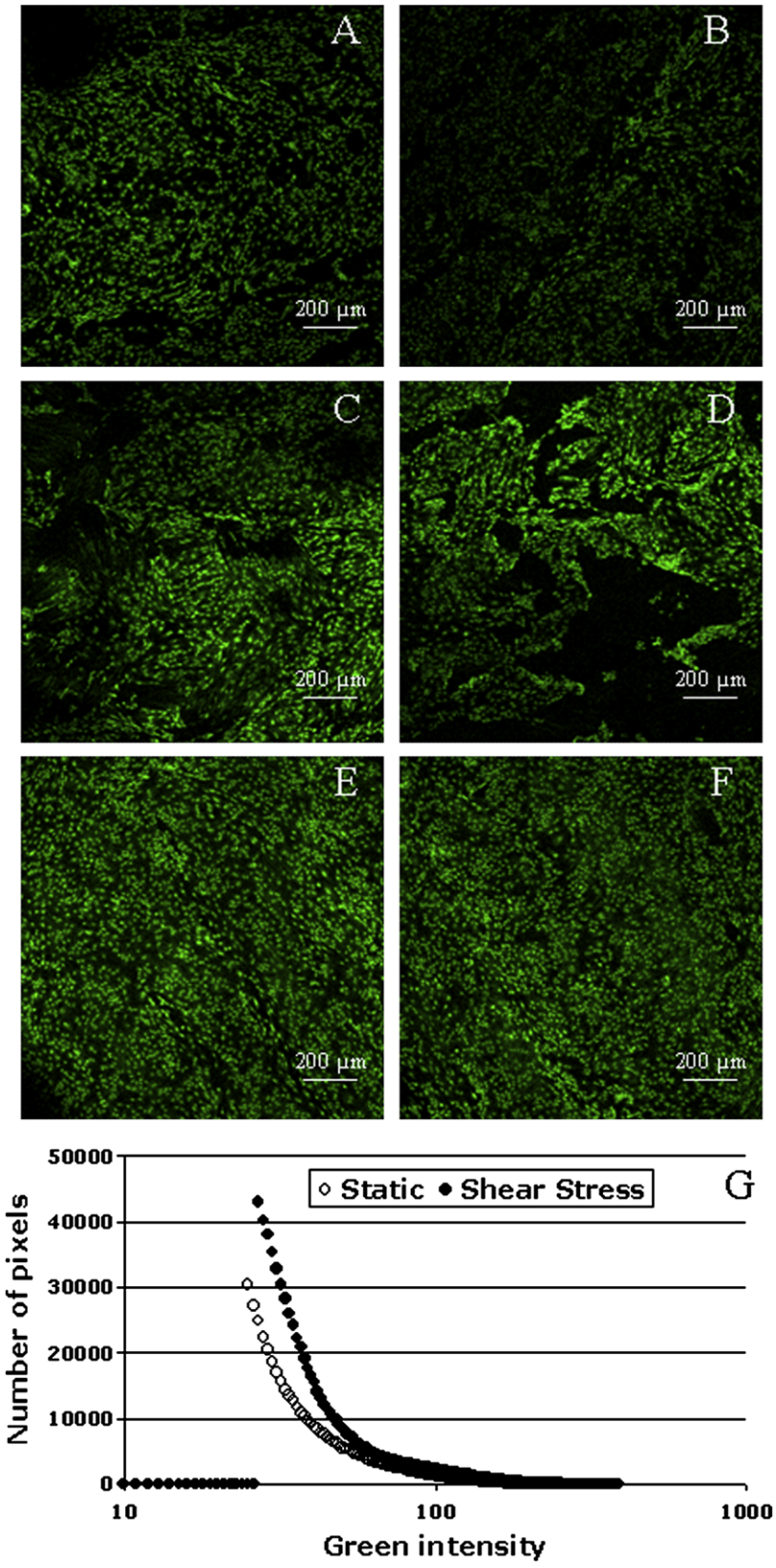

Fig. 4. Confocal laser scanning microscopy following incubation with Sytox green nucleic acid stain showing a PDEC monolayer before connecting cellularized prostheses in the hemodynamic bench (A,B), after 6 h in static conditions (C,D) and after 6 h of shear stress (E,F). (G) Quantitative analysis of fluorescence intensity.

sitivity [43]. In order to attain low permeability, the prosthesis POLYMAILLE® C was impregnated with collagen: the procedure, which involves cross-linking followed by irradiation, probably leads, under our conditions, to leach-

Table 2
Results of quantitative real-time RT-PCR obtained in PDECs seeded on POLYMAILLE® C grafts then submitted to arterial shear stress for 4 and 8 h ($n = 3$ per condition and time) and compared with corresponding static controls as ratios of shear stress over static conditions (means ± SEM).

	Ratio at 4 h	Significance	Ratio at 8 h	Significance
vWF	13 ± 10.73	NS	2.89 ± 2.16	NS
MMP1	1.54 ± 0.27	$p < 0.05$	1.43 ± 0.13	NS
VE-cadherin	1.65 ± 0.68	NS	1.31 ± 0.47	NS
KDR	1.82 ± 0.91	NS	1.44 ± 0.5	NS

vWF, MMP1, VE-cadherin and KDR are defined in the footnote of Table 1. NS, not significant.

ables that are able to alter cell metabolic activity at the two highest concentrations. This effect was not detectable for 10 and 1 vol.% diluted extract, nor for material extracts obtained after extensive washings (data not shown).

Pre-coating implant surfaces with protein may influence the biocompatibility of the implant materials so as to support cell coverage. Cell adhesion is a complex process depending on multiple interactions between surface topography, matrix receptors such as integrins and diverse cytoplasmic proteins. Collagen, a component of the extracellular matrix, contains biologically active sites such as RGD and DGEA sites, which are known to interact with and bind to integrins present on the cell surface [44]. Thus, the second experimental series was designed to determine whether the precoated surface provided a substratum that would allow cell attachment in the absence of serum. From our results, endothelial attachment appears to be related to CSD, with the absolute number of attached cells on patches being nearly fivefold higher at 24 h for CSD2 compared with CSD1. Although seeding densities ranging from 8×10^4 to $1.5 \times 10^6\ cm^{-2}$ have been reported [17,36,45–47], plating efficiencies have rarely been detailed in the literature. Anderson et al. [45] established that cell attachment was directly related to the number of cells available in the cell suspension medium, with a saturation cell density of approximately $3.5 \times 10^5\ cm^{-2}$ in the case of polytetrafluoroethylene grafts precoated with type I collagen. There is no consensus on the optimal seeding density [47].

Taking into account the PDEC growth pattern on vascular grafts, CSD2 is likely to represent the most suitable seeding density, leading to an estimated attachment density of approximately 5×10^4 cells cm^{-2}, which is sufficient to obtain total coverage by cells upon histological examination (Fig. 2). Such a confluency could be maintained for at least 36 h by day 7 in static conditions. It is well known that EPCs consist of two different subpopulations, termed early and late EPCs, which are phenotypically and morphologically distinct. Recently, it has been shown that their role in vascular network formation is also strikingly different [48,49]. It is presumed that the endothelial cells expanded in the present study are reasonably late outgrowth ECs, according to the time at which they appear in culture and their cobblestone morphology [29,30]. Interestingly, the statement by Igreja et al. [50] that EPC adhesion and differentiation are impaired and largely inhibited on a collagen type I matrix could not be verified in our study.

Aside from obtaining a confluent EC monolayer on the lumen of a vascular graft, EC retention as well as cell function on exposure to flow are other important factors to consider. In this regard, Feugier et al. [46] measured the cell coverage and resistance to shear stress of human umbilical vein endothelial cells (HUVECs) seeded onto commonly used type I/III collagen-coated materials. Fluid shear stress exerts many effects on vascular ECs and plays an important role in maintaining the homeostasis of blood vessels. Consistent with Wong et al.'s study [47] and that of Sreerekha et al. [51], our results exhibit an effective cell retention on polyethylene terephthalate. In addition, it was shown in 1997 [52] that confluent cultures of ECs without application of hemodynamic forces undergo morphological changes that indicate apoptotic cell death, which can be prevented by exposure to laminar flow. However, it remains to be proven whether the morphological features observed in Fig. 4C and D, as well as the graph in Fig. 4G, are related to the lack of hemodynamic forces, a general endothelial feature [53].

In vitro findings have shown that shear stress modulates EC gene expression [54,55]. It is noticeable that, in the latter study, cell monolayers were investigated once mounted in flow channels. As proposed by Vara et al. [56], studying gene expression in ECs seeded on tubular conduits is superior to studying them on flat glass slides. Thus, we investigated, via changes in mRNA levels, the ability of PDECs to be regulated by arterial shear stress when they colonize tubular surfaces. Four genes were explored: one was related to the hemostatic balance (*vWF*); two were related to the mechanosensory complex, that is, *Flk-1/KDR* and *VE-cadherin*, which confer responsiveness to flow [25]; and one was related to extracellular matrix (MMP1). The rationale for this choice was as follows: (i) vWF, VE-cadherin and KDR are all specific markers of endothelial cells, currently used either for characterization or endothelial isolation, and present or expressed in PDEC; (ii) MMP1 (interstitial collagenase) belongs to a family of endopeptidases able to degrade extracellular matrix (ECM) components [57], and its expression is up-regulated by arterial hemodynamic conditions [54,58]. Thus, the MMP1 gene expression was explored in our study as a positive control. In accordance with the literature on other experimental conditions and with other human EC types [54,59,60], we report here the significant up-regulation of MMP1 with as little as 4 h of shear stress in PDECs. Although a direct comparison may not be possible due to species and cell type differences, it is interesting to report the study of Long et al. [61]: muscle-derived stem cells were seeded onto small intestinal submucosa and the release of MMP1 in response to initial cell seeding and the subsequent breakdown of collagen fibers were the mechanisms suggested to explain the

increased compliance. There is evidence that MMP1 may be involved in EC migration and wound healing, and that increase in MMP1 expression by shear stress would be beneficial for EC migration and wound healing [54]. Hence, we suggest that the increase in MMP1 expression by shear stress in our study could be linked to ECM reorganization and EC remodeling. Moreover, it has allowed us to show that PDECs are responsive to shear stress.

In our study, vWF mRNA expression was not significantly regulated by shear stress; nor was it for Galbusera et al. [62], though it was for Hough et al. [63]. However, PDECs on both static and shear-stressed vascular grafts expressed vWF, indicating that ECs were functioning properly in both cases.

In the same way, we were not able to demonstrate any significant changes in VE-cadherin and KDR mRNA expression. Kondapalli et al. [64] also found no significant change in VE-cadherin mRNA expression in human coronary artery EC under laminar shear stress, whereas others [60] found a down-regulation in human aortic ECs exposed to atheroprotective flow conditions. In contrast, Yamamoto et al. [65] demonstrated a markedly increase in protein and mRNA expression of both VEGFR-2 and VE-cadherin.

Different authors have shown the up-regulation of KDR mRNA expression in HUVECs submitted to laminar shear stress ranging from 5 to 45 dyn cm^{-2} [54,66,67]. However, these assumptions should probably be tempered by the composition of the underlying matrix cell substrate. Indeed, from a signaling pathway point of view, vitronectin has been reported to positively regulate KDR signaling, whereas collagen I down-regulates VEGFR-2 activation [68]. Furthermore, Orr et al. [69] presented evidence that the underlying matrix composition regulates shear-stress-induced NFκB activation: fibronectin and fibrinogen support activation, whereas laminin and collagen do not. The integrins, through their specific and dynamic conditions with ECM ligands, are known to transmit mechanically initiated signals into the cell to trigger the intracellular signal transduction pathways for the modulation of gene expression and cellular functions.

In addition, flow differentially influences endothelial adhesion molecules and transcription factor expression, depending on the vascular bed origin [30]. Experimental complexities are plausible explanations for discrepancies between some of our results and those in the literature, ranging from differences in cell derivation (cell origin, harvesting technique) to technical issues (absence of serum during flow experiments, type and duration of shear stress). In this respect, the observations of Andersson et al. [70] reinforce the influence of experimental conditions. Comparing the expression profile data generated by different laboratories in non-comparable in vitro conditions makes a true consensus expression profile difficult to achieve, since no two flow experiments are ever truly the same, especially for PDECs, which have not yet been studied extensively in flow conditions.

Moreover, some limitations are appropriate regarding the conduct of this study. The first is the use of a single type of ECs – PDECs – for these analyses. However, other investigators have demonstrated that they harbor a number of phenotopic characteristics of mature ECs. A second limitation is the use of PDECs in a no-flow environment as controls to be compared with laminar shear stress. Even if there is no ideal in vitro system to mimic the spectrum of flow regimes, it will be interesting to examine the response of these cells to other regimes, e.g. turbulent or disturbed flows. Third, an extended period of shear stress could be investigated, as could other genes. Being limited practically by the in vitro model to the duration of our experiments, we chose to measure gene expression at 8 h after shear stress changes form an established arterial baseline. This choice was based on indications that gene expression appears to reach steady-state values within this time frame [71].

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