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Improving bacterial cellulose for blood vessel replacement: Functionalization with a chimeric protein containing a cellulose-binding module and an adhesion peptide

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ABSTRACT

Chimeric proteins containing a cellulose-binding module (CBM) and an adhesion peptide (RGD or GRGDY) were produced and used to improve the adhesion of human microvascular endothelial cells (HMEC) to bacterial cellulose (BC). The effect of these proteins on the HMEC–BC interaction was studied. The results obtained demonstrated that recombinant proteins containing adhesion sequences were able to significantly increase the attachment of HMEC to BC surfaces, especially the RGD sequence. The images obtained by scanning electron microscopy showed that the cells on the RGD-treated BC present a more elongated morphology 48 h after cell seeding. The results also showed that RGD decreased the in-growth of HMEC cells through the BC and stimulated the early formation of cord-like structures by these endothelial cells. Thus, the use of recombinant proteins containing a CBM domain, with high affinity and specificity for cellulose surfaces allows control of the interaction of this material with cells. CBM may be combined with virtually any biologically active protein for the modification of cellulose-based materials, for in vitro or in vivo applications.

1. Introduction

Cardiovascular disease is the leading cause of mortality in Western countries. Surgical bypass with autologous grafts remains the most used treatment, saphenous veins and mammary arteries being preferably used. However, many patients do not have suitable vessels, due to pre-existing vascular disease, amputation or previous harvest for prior vascular procedures. Moreover, a second surgical procedure is needed to obtain the vessel [1,2]. For the reconstruction of arteries of large caliber currently available synthetic grafts (e.g. Dacron, ePTFE and polyurethane) offer a reasonable solution and proven clinical efficacy. However, for small sized (<6 mm) grafts these materials generally give poor performance, due to anastomotic intimal hyperplasia and surface thrombogenicity [3–5]. This scenario prompts the search for new materials suitable for the effective replacement of small blood vessels.

Bacterial cellulose (BC) produced by *Acetobacter* spp. is a biomaterial that has gained interest in the field of tissue engineering due to its unique properties. BC has been studied by several research groups as a scaffold for cartilage [6–8], wound dressing [9,10], dental implants [11–17], nerve regeneration [18,19] and vascular grafts [18,20–23]. The in vivo biocompatibility of BC was also evaluated in a study conducted by Helenius and colleagues [24].

Many strategies have been pursued to improve the compatibility and effectiveness of vascular grafts, through the production of unreactive surfaces, the surface modification of existing synthetic grafts (e.g. modifying surface properties and the incorporation of biologically active substances) and coating with autologous cells [4]. Seeding the graft surface with endothelial cells [25] is a promising approach; this mimicks the native vessel, thereby decreasing thrombosis. However, the high loss of endothelial cells on the restoration of blood flow after implantation presents a major challenge [4,26,27]. The rate and quality of endothelialization of a synthetic vascular graft depends on the interaction of endothelial cells with these cardiovascular materials. Several approaches have been attempted to increase endothelial cell adhesion to typically non-adhesive polymeric biomaterials used for synthetic vascular grafts [28]. One such approach involves pre-coating with endothelial cell-specific adhesives. The tripeptide Arg–Gly–Asp (RGD), an amino acid sequence found in many adhesive plasma and extracellular matrix proteins, has been used to enhance cell adherence. Binding of cells to the RGD sequence occurs via integrin receptors on the cell membrane. An improvement in the biocompatibility and performance of BC – envisaging its use as small diameter vascular grafts – by enhancing adhesion to human microvascular endothelial cells (HMEC-1) was attempted in this work by coating BC with adhesion peptides.

Many strategies have been developed to modify the materials used as synthetic grafts (e.g. Dacron, ePTFE and polyurethane). The adsorption of active substances like heparin, RGD, albumin–

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heparin conjugates, dipyridamole have little or no effect, due to the coatings being washed away [27]. In a previous work we described the production of recombinant proteins containing adhesion sequences fused to a CBM (cellulose-binding module) [29]. For artificial grafts based on cellulose the use of a CBM (exhibiting high affinity and specificity for cellulose surfaces) that can be combined with virtually any biologically active protein is an important strategy to avoid loss of the biological agents coating the graft.

2. Materials and methods

2.1. Cell culture assays

Human microvascular endothelial cells (HMECs) (kindly provided by Dr. João Nuno Moreira, Coimbra University) were used between passages 13 and 22. HMECs were cultured in RPMI 1640 medium (Invitrogen Life Technologies, UK) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, UK), 1% penicillin/streptomycin (Invitrogen Life technologies, UK), 1.176 g l^{-1} sodium bicarbonate, 4.76 g l^{-1} HEPES, 1 ml l^{-1} EGF and 1 mg l^{-1} hydrocortisone (>98% purity, Sigma, Portugal) and maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Cell attachment, proliferation and viability

2.2.1. Production and purification of recombinant proteins

The recombinant peptides (RGD–CBM, RGD–CBM–RGD, GRGDY–CBM, GRGDY–CBM–GRGDY) have been previously cloned in *Escherichia coli* and their production and purification were conducted as previously described [29].

2.2.2. Production of bacterial cellulose and coating with the recombinant peptides

Gluconacetobacter xylinus (ATCC 53582 and DSMZ 46604) purchased from the American Type Culture Collection and from the German Collection of Microorganisms and Cell Cultures were grown in Hestrin–Schramm medium, pH 5.0. The medium was inoculated with the culture, added to 24- or 96-well polystyrene plates (1 or 0.2 ml per well, respectively) and incubated statically at 30 °C for 5 (ATCC 53582) or 10 days (DSMZ 46604). BC pellicles were purified by 2% SDS treatment at 60 °C for 12 h followed by 4% NaOH at 60 °C for 90 min. Samples were autoclaved and stored in phosphate-buffered saline (PBS), pH 7.4, at 4 °C prior to use. The pellicles produced by the DSMZ 46604 strain (BC-L) had a thickness of about 0.5 mm, while the pellicles produced by the ATCC 53582 strain (BC-H) were approximately 3 mm thick. The recombinant proteins CBM and RGD–CBM (0.25 mg protein per membrane) were left adsorbing to BC for 12 h at 4 °C. Then, the membranes were washed with PBS.

2.2.3. HMEC-1 adhesion and proliferation

The mitochondrial activity of the cultured cells was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) colorimetric assay, which is related to cell viability. BC-H or BC-L membranes treated with the recombinant peptides were added to the wells of 24-well polystyrene plates. The BC sheets were produced in similar 24-well polystyrene plates, such that they fitted tightly in the wells, completely covering the bottom surface. The HMEC-1 cells were seeded on the BC at a density of 12×10^4 cells $well^{-1}$ in RPMI medium without serum. The plates were incubated at 37 °C in a 5% CO_2, 95% humidified air atmosphere. Two hours after addition of the cells the wells were washed with PBS and RPMI with 10% FBS was added. MTS assay of the adsorbed HMEC-1 cells was carried out to evaluate adhesion of the cells after 2 h and proliferation 24 and 48 h and 7 days after cell seeding. The results were obtained from at least three separate assays, each one done in triplicates.

In order to evaluate the effect of RGD on the rate of cell adhesion a similar assay was performed at 15, 30, 60, 90 and 120 min after cell seeding, with the non-adherent cells being washed out before carrying out the MTS assay.

2.2.4. Live and dead assay

The viability of the cells coating the cellulose (BC-L), treated or not with the recombinant peptides, was also analyzed using a Live/Dead® viability/cytotoxicity kit for mammalian cells (Invitrogen, UK). This kit provides a two-color fluorescence cell viability assay, based on the simultaneous determination of live and dead cells with two probes that measure intracellular esterase activity and plasma membrane integrity. This assay employs calcein, a polyanionic dye, which is retained within living cells, producing a green fluorescence. It also employs an ethidium bromide homodimer dye (red fluorescence), which can enter the cells through damaged membranes, binding to nucleic acids, but is excluded by the intact plasma membrane of living cells. The experiment was developed as described for the MTS assay. Fluorescence microscopy observations of the cells were carried out after 24 h incubation. Cells seeded on polystyrene were used as a positive control (living cells), and cells further treated with 70% methanol for 30 min were taken as a negative control (dead cells). The Live/Dead assay was also used to determine apoptosis (qualitatively), in combination with the TUNEL assay (quantitatively). Samples were visualized and imaged using an Olympus BX51 fluorescence microscope (Olympus Portugal SA, Porto, Portugal).

2.3. Morphological analysis by fluorescence and scanning electron microscopy (SEM)

BC-L membranes treated with recombinant peptides were seeded with cells as previously described. For fluorescence microscopy the membranes were washed with pre-warmed PBS 14 days after cell seeding. Then the cells were fixed in 4% formaldehyde (Pierce, Rockford, IL) in PBS, permeabilized with acetone (Sigma) at −20 °C and stained with Alexa Fluor 546-phalloidin (Molecular Probes). Nuclei were visualized by staining with DAPI. Microscopy observations were performed using an Olympus BX51 (Olympus Portugal SA) fluorescence microscope. Fluorescence microscopic observations were carried out only on the BC-L membranes, which allowed proper visualization of the cells due to their thinness. For SEM microscopy the medium was removed 48 h after cell seeding and the BC pellicles were washed twice with PBS. Next, 1 ml of 2.5% glutaraldehyde in PBS was poured into each well and the materials were maintained at room temperature for 1 h, in order to fix the cells on the membrane. Afterwards the membranes were rinsed with distilled water, dehydrated by successive immersion in a series of aqueous ethanol solutions (55, 70, 80, 90, 95 and 100 vol.%) for 30 min each, and allowed to evaporate at room temperature. The surfaces of the membranes with adherent cells were observed by SEM (Leica S360) after gold sputtering.

2.4. TUNEL assay for cell apoptosis

HMEC-1 cells (12×10^4 cells $well^{-1}$) were seeded on BC-L as described for the MTS assay and after 24 h incubation the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) assay, which examines DNA strand breaks during apoptosis, was performed using an In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions and as previously described [30].

To facilitate counting of the total number of nucleus, the cells were also stained with DAPI.

2.5. Cell invasion

To evaluate the effect of RGD on the migration of endothelial cells through the BC a migration chamber and an attractant were used to stimulate cell growth into the BC. The migration chamber consisted of cell culture inserts with a membrane pore size of 8.0 μm in a 24-well plate (BD Biocoat™ Matrigel™ Invasion Chamber, BD Biosciences, Franklin Lakes, NJ). Initially, HMEC-1 cells were seeded onto BC-L (treated with the recombinant peptides) at a density of 2.5×10^4 cell well^{-1} in RPMI medium without serum. After 4 h 10% FBS was added to the wells. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air for 24 h. The next day the cell-coated BC pellicles were transferred to the invasion chamber containing medium with 2% FBS. To stimulate the cells to migrate into the cellulose 20% FBS were added to the cell culture medium in the wells. Cell cultures were incubated for 72 h. After the conclusion of the experiments the Matrigel membranes were removed from the inserts with a scalpel. The cellulose and Matrigel membranes were fixed and stained with methanol–DAPI solution and observed by fluorescence microscopy. The cells that migrated from the cellulose into the Matrigel were counted.

2.6. Angiogenesis

BC-L pellicles produced in a 24-well polystyrene plate were treated with the recombinant proteins and coated with HMEC cells (4×10^4 cells well^{-1}) in serum-free medium for 2 h. Then 10% FBS was added to the wells. The pellicles were incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air. Afterwards the medium was removed and fresh medium with 10% serum was added. The plates were incubated for 4 days. To evaluate the effect of the recombinant peptides on the morphology and assembly of endothelial cells into capillary-like structures when cultured on BC pellicles, the cord-like structures were observed qualitatively using a Leica DM IL inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.7. Immunocytochemistry

The cells were grown on BC treated with the recombinant peptides (RGD–CBM or CBM) or buffer for 14 days, then fixed with methanol at −20 °C. To avoid non-specific interactions the cellulose membranes were blocked with 4% bovine serum albumin in PBS. The primary antibody was von Willebrand factor (vWF) (1:100) (Chemicon, Hofheim, Germany) and the secondary antibody FITC-conjugated anti-rabbit (1:1000) (Santa Cruz Biotechnology, Santa Barbara, CA). The nuclei were counterstained with DAPI (Sigma Aldrich, Portugal). Cells were observed by fluorescent microscopy (Nikon Eclipse 50i, Japan). The endothelial specificity of the cells was also verified by the uptake of DiL-labelled acetylated low density lipoprotein (Biomedical Techologies, USA) another specific marker for these cells.

2.8. Statistical analyses

All experiments were performed in triplicate. Quantifications are expressed as means ± SD of three independent experiments. The statistical significance of differences between various groups were evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni test.

3. Results

The results of the MTS assay demonstrate that the recombinant peptides containing adhesion sequences were able to significantly increase the attachment of HMEC to BC-H surfaces (Fig. 1). Two hours after cell seeding approximately 140–150% and 60–80% more cells adhered to BC treated with the peptides containing the RGD and GRGDY sequences, respectively, when compared with untreated BC-H. The results demonstrate that the peptides containing RGD sequence had a stronger effect than the peptides containing GRGDY sequences. Moreover, it seems that the presence of a second adhesion sequence at the C-terminus did not significantly enhance the effect of the recombinant peptides when compared with peptides containing only one copy of the sequence. Moreover, the results indicate that adsorption of the CBM peptide on BC-H slightly decreased cell adhesion (Fig. 1) by 14%. When the assay was developed with BC-L membranes coated with peptides containing one or two RGD copies approximately 108% and 77% more cells adhered to the material than to untreated BC-L. The proteins containing the GRGDY sequence promoted an increase of only 22–40% in cell adhesion (Fig. 2). Fig. 3 shows that the improvement in cell attachment was significant as early as 15 min after cell seed-

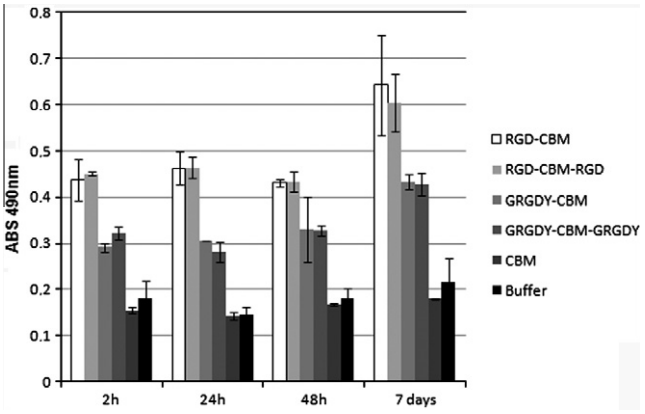

Fig. 1. MTS assays of HMEC-1 cultures on BC–H pellicles treated with the recombinant proteins (CBM, RGD–CBM, RGD–CBM–RGD, GRGDY–CBM and GRGDY–CBM–GRGDY) and buffer. The MTS assay was developed at 2, 24 and 48 h and 7 days after cell addition. Results are expressed as absorbance values at 490 nm.

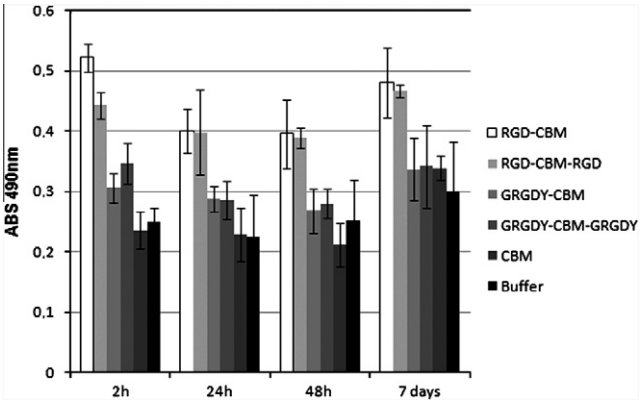

Fig. 2. MTS assays of HMEC-1 cultures on BC-L pellicles treated with the recombinant proteins (CBM, RGD–CBM, RGD–CBM–RGD, GRGDY–CBM and GRGDY–CBM–GRGDY) and buffer. The MTS assay was developed at 2, 24 and 48 h and 7 days after cell addition. Results are expressed as absorbance values at 490 nm.

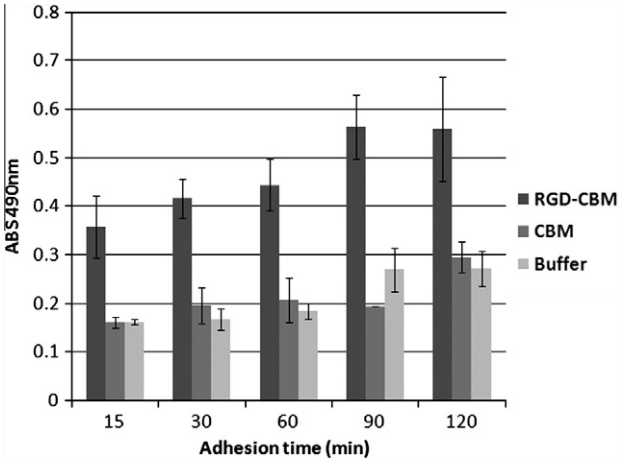

Fig. 3. MTS assays of HMEC-1 cultures on BC-H pellicles treated with CBM, RGD–CBM and buffer. The MTS test was developed at 15, 30, 60, 90 and 120 min after addition of cells. Results are expressed as absorbance values at 490 nm.

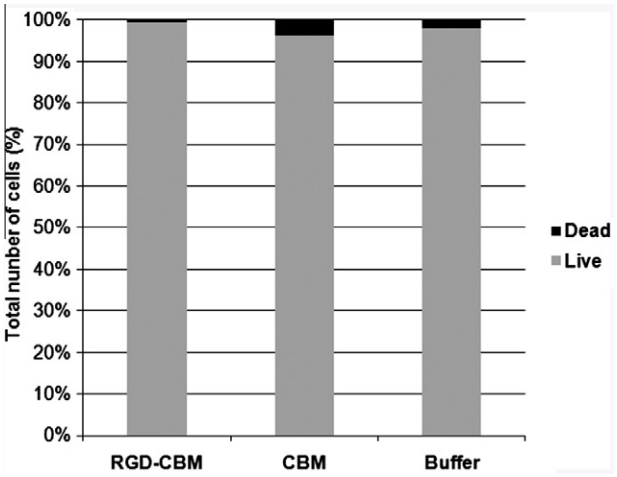

Fig. 5. Apoptosis was quantitatively evaluated by the TUNEL assay. HMEC cells were seeded on BC–L and after 24 h incubation a TUNEL assay was performed. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL stained cells and DAPI stained nuclei in each culture. Experiments were repeated three times with identical results.

ing. No proliferation was detected 24 and 48 h following cell seeding (Figs. 1 and 2), irrespective of the BC membrane or treatment used. However, after 7 days proliferation was noticeable on BC-H treated with the RGD- and GRGDY-containing peptides (in contrast to BC-H treated with CBM or buffer), while no proliferation was visible when cells were cultured on BC-L.

To estimate the viability of cells on the protein-coated BC a Live/Dead assay was performed 24 h after cell adhesion. The fluorescence images obtained show that, irrespective of the treatment, the cells remained viable on the BC pellicles (Fig. 4). The TUNEL assay results corroborated the Live/Dead assay, showing no significant differences between BC pellicles treated with the recombinant peptides (RGD–CBM and CBM) when compared with the control (buffer) (Fig. 5).

We next investigated whether RGD affected HMEC invasion capacity using a double chamber assay. The cells were seeded on the BC-L pellicle treated with the RGD or CBM sequence or buffer. The number of migrating cells was then quantified through the double chamber assay, using serum at 20% as a chemoattractant. In comparison with the controls, RGD decreased the in-growth of HMEC cells through BC. Four and 2.4-fold increases in the number of cells migrating through the membrane were obtained for the BC treated with buffer and CBM, respectively, taking as a reference BC treated with RGD (Fig. 6).

Optical microscopy indicated that RGD stimulated the formation of cellular cord-like structures at an earlier stage as compared with the other groups. These findings showed that 24 h after seeding most of the cells had a round shape in all groups (data no shown). However, after 96 h the cells on the RGD-treated BC were more elongated than those on the buffer control, starting to form cord-like structures, while the cells in the CBM group remained round shaped (Fig. 7). In fact, the structure shown in Fig. 7d, obtained by fluorescent microscopy, was found only on the BC surfaces treated with RGD. Details of the morphology of the cells 48 h after cell seeding were obtained by SEM. Cells on the RGD-treated BC presented an elongated shape; in fact, as can be seen in Fig. 8a, most of the cells were so elongated that they were hardly noticeable by SEM, unlike cells observed on the untreated or CBM-

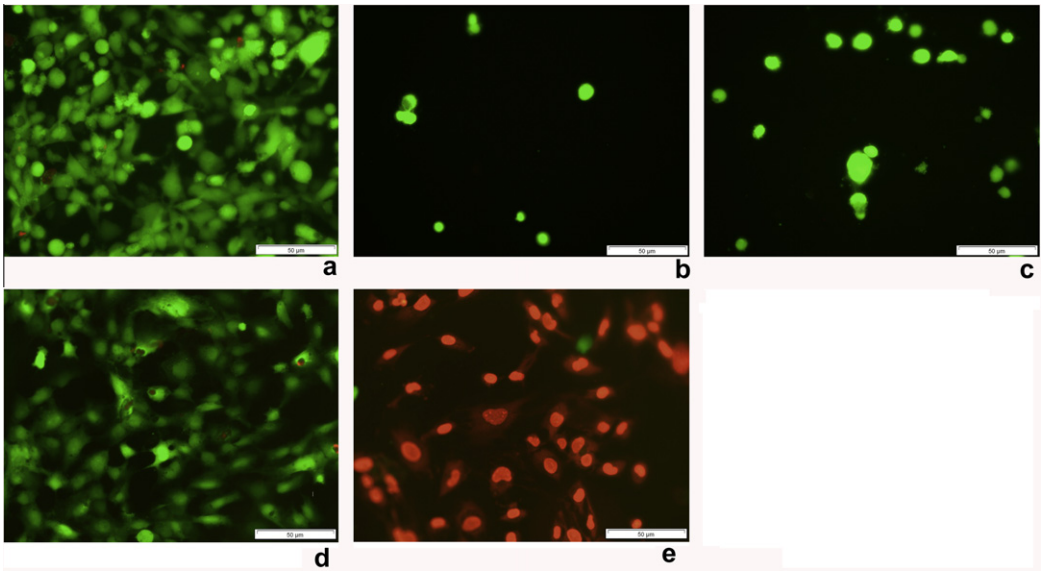

Fig. 4. Fluorescence photographs of endothelial cells stained using a Live/Dead® viability/cytotoxicity kit for mammalian cells. Live cells are stained green, dead cells red. BC-L treated with (a) RGD–CBM, (b) CBM or (c) buffer. Controls contained (d) live and (e) dead cells on polystyrene. Images were acquired using a 40× objective (scale 50 μm).

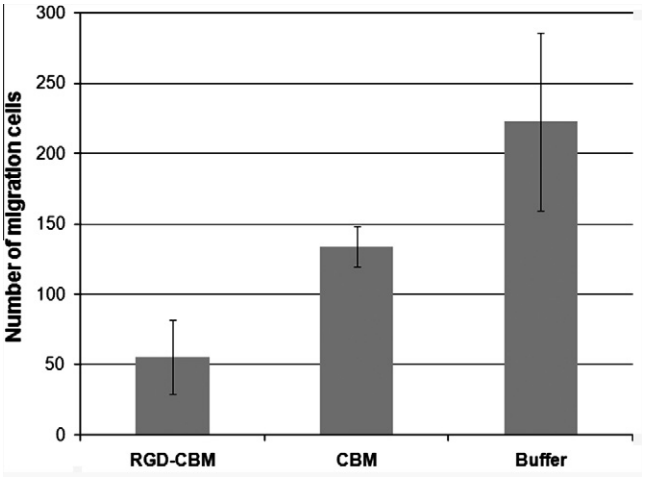

Fig. 6. Effect of RGD on HMEC cell invasion of bacterial cellulose pellicles. Invasion was quantified in a double chamber assay using medium complemented with 20% FBS as a chemoattractant. Bars represent the number of invasive cells.

treated BC, where most remain round (Fig. 8). vWF expression is a widely used criterion in defining the endothelial cell phenotype [31–33], thus to determine whether HMECs maintain this endothelial phenotype characteristic after 14 days culture on BC vWF expression was evaluated. The immunocytochemistry results show that cells grown on BC treated with the recombinant peptides or buffer maintained their positive staining for vWF (Fig. 9).

4. Discussion

G. xylinus constructs a BC pellicle with a denser and flatter surface on one side and a gelatinous layer on the other [18]. In this study all the experiments were conducted on the denser side of both BC-H and BC-L, because a smooth surface, being similar to the basal membrane of the luminal side of blood vessels, is preferable for the attachment of endothelial cells [20]. Analysis by SEM showed that *G. xylinus* ATCC 53582 produced a thicker and more compact cellulose pellicle than strain DSMZ 46604 (data not shown). Therefore, the BC-H pellicle presents a smoother surface than BC-L. This may lead to differences between BC-H and BC-L in the adhesion and proliferation of cells in the MTS test. The results of the attachment assay were similar to those obtained in our previous work, when fibroblasts were seeded on BC produced by strain ATCC 53582 coated with adhesion peptides [29]. In that previous work RGD improved the adhesion of fibroblasts to cellulose, while the presence of a second RGD did not enhance the effect of the recombinant peptide, probably because the RGD sequence at the C-terminus of the peptide was not exposed in such a way as to be recognized by integrins. However, unlike the results with endothelial cells, the GRGDY sequence had no effect on the adhesion of fibroblasts. Apparently, microvascular endothelial cells adhere more strongly than fibroblasts to recombinant peptides containing RGD sequences. Indeed, endothelial cells may have substantially more $\alpha_v\beta_3$ integrin than fibroblasts [34]. The results also demonstrate that pre-coating BC with the RGD-containing peptides decreased the incubation time required for adsorption. A short incubation period is particularly important in single stage seeding as the incubation time is kept to a minimum to fit within the time frame of the surgical procedure [26].

Several works have been developed to improve the interaction of cells with BC [21,29,35,36]. However, only a few have studied the migration and in-growth of cells on BC [20,21,6,37]. The migration of cells is mainly mediated by integrins, a diverse family of glycoproteins that form heterodimeric receptors for extracellular matrix (ECM) molecules. During migration cells project lamellipodia that attach to the ECM and simultaneously break existing ECM contacts at their trailing edge. This allows the cell to pull itself forward. Integrins are essential for cell migration and invasion, not only because they directly mediate adhesion to the ECM, but also because they regulate intracellular signaling pathways that control cytoskeleton organization, force generation, gene transcription and survival [38].

Endothelialization may either be developed ex vivo or post-implantation, stimulating endothelial cells (from tissues adjacent

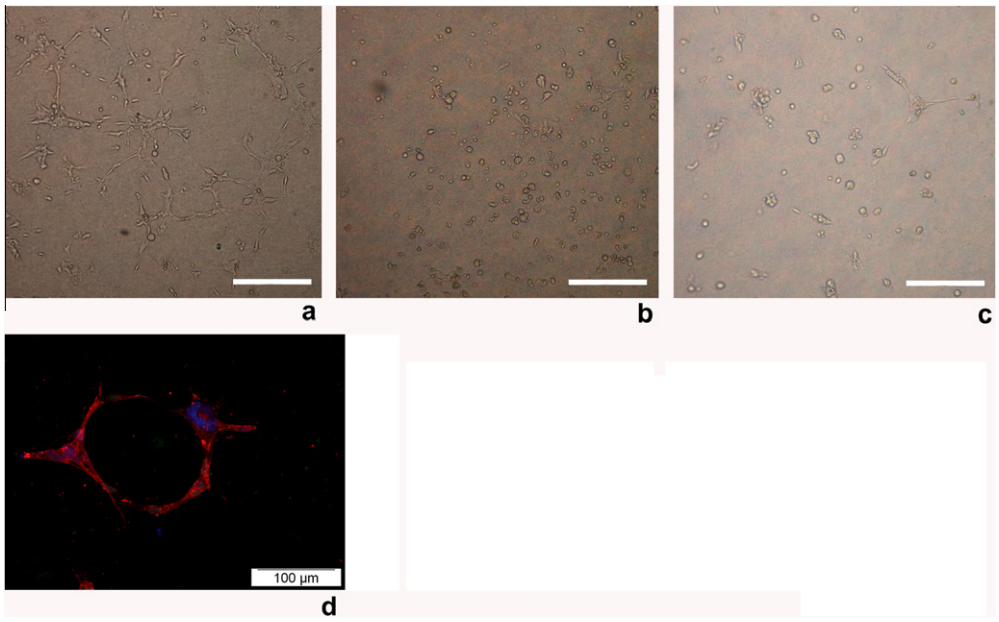

Fig. 7. (a–c) Optical micrographs showing the effect of RGD on the assembly of endothelial cells into capillary-like structures: (a) BC-L treated with RGD–CBM; (b) CBM; (c) buffer. Images were acquired using a 20× objective (scale 200 µm). (d) Fluorescent microscopy image showing HMECs cells cultured for 14 days on BC-L pellicles treated with RGD–CBM recombinant protein. Nuclei were visualized by staining with DAPI (blue) and F-actin with Alexa Fluor 546-phalloidin (red). Images were acquired using a 20× objective (scale 100 µm).

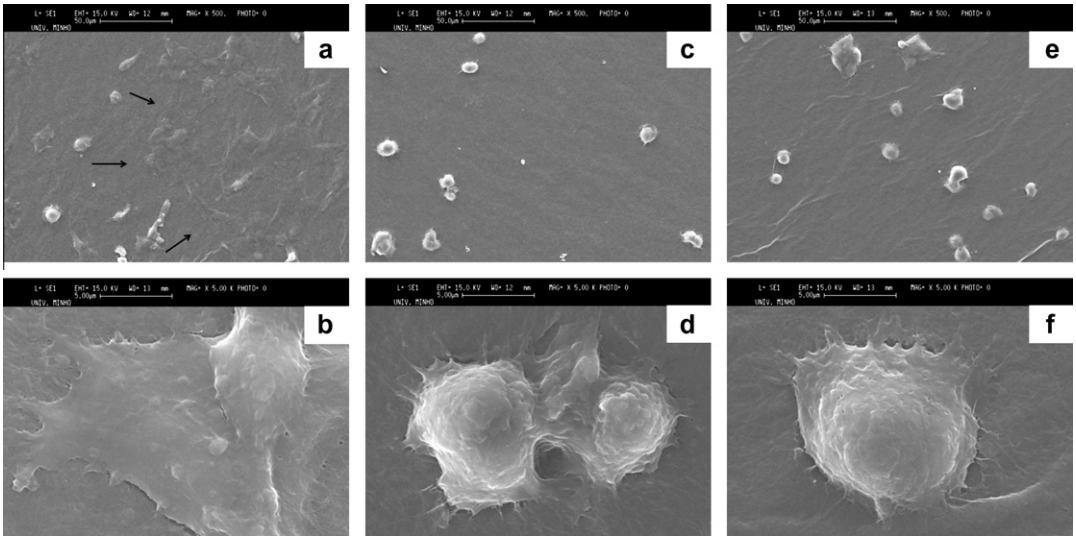

Fig. 8. SEM micrographs of bacterial cellulose. BC treated with: (a and b) RGD–CBM; (c and d) CBM; (e and f) buffer. The arrows mark cells with an elongated morphology. (a, c, and e) Scale 50 μm; (b, d, and f) scale 5 μm.

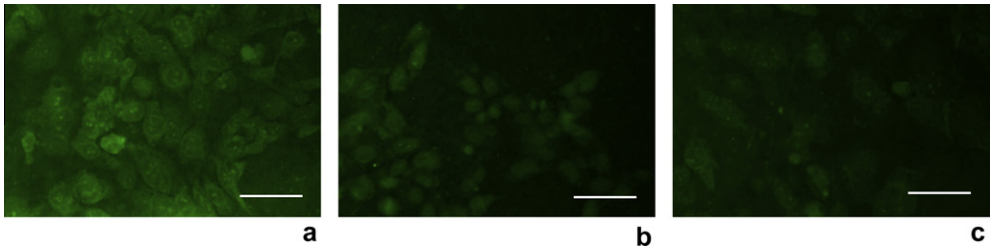

Fig. 9. Immunocytochemical analyses using anti-vWF antibody. The results showed that HMEC cells cultured for 14 days on BC-L treated with recombinant proteins or buffer stained positively for vWF: (a) RGD–CBM; (b) CBM; (c) buffer. Images were acquired using a 20× objective (scale 100 μm).

to the anastomosis or from the circulation) to adhere and proliferate on the graft. The rate and quality of vascular graft endothelialization depends on cell–material interaction, leading to such as adhesion and migration. Several studies have shown that cell adhesiveness to the substratum modulates cell migration on surfaces coated with ECM proteins [25,39–41]. Our results have shown that a small number of cells migrated through the cellulose when compared with the rather large number of cells added (2.5×10^4 cells well^{-1}). During invasion cells release proteases that degrade and remodel the ECM, promoting cell passage through to the stroma and entrance into the new tissue [38]. However, animal cells cannot degrade cellulose [24] and in order to migrate in a fibrous hydrogel such as BC the cells must push the nanofibrils aside when migrating into the cellulose network [20]. Probably the time course of the experiment (72 h) was too short to enable cells to migrate through a BC pellicle of ~0.5 mm thickness. Nevertheless, the results obtained allow the observation that the migration of endothelial cells on BC was decreased by the presence of RGD. Since adhesion involves receptor/ligand binding, cell migration can be regulated by controlling cell integrin expression, integrin–ECM binding affinity or substratum ECM surface density. However, if other stimuli are added, such as growth factors that affect signaling processes of the cell, the migration/adhesion relationship can be dramatically altered [42,43]. The migration rates of cells are influenced by chemical and physical interaction with the surface of the material. Previous research has shown that cell migration capacity presents a biphasic behavior depending on the attachment strength. Optimal migration speed can be achieved with intermediate strengths of adhesiveness, since when adhesion to the substratum is weak no traction occurs, so that movement is impossible and the cell spreads poorly. On the other hand, with strong adhesion the cell is well-spread and immobilized, so dynamic disruption of cell–substratum attachments is difficult and movement again does not occur [28,39,40,44,45]. The CBM used in this work had a high affinity for cellulose and was adsorbed in a specific and very stable way. Probably the amount of protein used in the experiments was enough to saturate the surface of the cellulose pellicle with RGD-containing peptides, resulting in a very high affinity of the cells for the substratum and negatively affecting migration through the BC. Saturation of the surface of the cellulose pellicle with RGD-containg peptides is corroborated by the results of our previous work [29]. In order to enhance endothelialization of BC vascular grafts it is important to promote not only the adhesion of endothelial cells, but also to allow migration through the material. The treatment used in this work greatly improved adhesion, however, migration was negatively affected by the presence of RGD, because the affinity of HMECs for the material surface became to strong. However, it is probably possible to improve migration of the cells on BC by optimizing the concentration of RGD-containing peptide, in an attempt to reach a compromise between adsorption and migration. However, longer experiments are needed to better assess the effect of RGD on cell migration through BC.

The effect of RGD on HMEC morphology was observed by SEM. The cells on the RGD-coated BC exhibited a more elongated, flattened morphology, while those on "bare" BC were

round (Fig. 8a and b). The more extended morphology of HMECs upon interaction with the adhesive peptides is likely driven by the greater number of focal contacts between integrins and RGD-containing peptides linked to the BC surface. It is well known that a critical RGD density is essential for the establishment of mature and stable integrin adhesions, which, in turn, induce efficient cell migration, spreading and formation of focal adhesions [46–49].

During angiogenesis cells must adhere to one another and to the ECM to construct and extend new microvessels [50]. Angiogenesis depends not only on growth factors and their receptors, but is also influenced by receptors for ECM proteins. Our results show that the RGD-containing recombinant peptide (RGD–CBM) stimulated the early formation of cellular cord-like structures on BC when compared with BC treated with a recombinant peptide without the adhesion sequence (CBM) or buffer. HMEC-1 cells express $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins [51] that can bind an array of ligands, such as vitronectin, fibronectin, vWF, fibrinogen, osteopontin, thrombospondin and RGD-containing peptides [50,52]. Moreover, these two complexes have also been identified as having an especially interesting expression pattern among vascular cells during angiogenesis and vascular remodeling.

Immunocytochemistry results showed that cells grown on BC maintained their positive staining for vWF. This glycoprotein is one of the various secretory and membrane-bound molecules produced by the endothelium. vWF mediates the interaction of platelets with damaged endothelial surfaces at sites of vascular injury and has long been favored as an endothelial cell marker, with expression of this factor being highly restricted to endothelial cells, platelets and megakaryocytes [33].

In the current scenario of regenerative medicine there is a great demand for the production of new materials appropriate for small diameter blood vessel replacements. In this work BC, a promising cardiovascular biomaterial, was successfully functionalized. The strategy used was aimed at improving microvascular cell adhesion to BC, through recombinant peptides containing adhesion sequences and a CBM. For artificial grafts based on cellulose the use of a CBM (exhibiting a high affinity and specificity for cellulose surfaces) is an excellent feature, as a CBM can be combined with virtually any biologically active protein and used to modify cellulose-based materials. The chimeric peptides were able to enhance endothelial cell adhesion to BC and stimulate angiogenesis. However, the in-growth of cells into cellulose was decreased. We believe that improved migration of the cells on BC will be achieved with intermediary concentrations of the peptides used in this work.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 4, 7 and 9, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.04.023.

References

[1] Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. Circ Res 2006;98(1):25–35.
[2] Wang X, Lin P, Yao Q, Chen C. Development of small-diameter vascular grafts. World J Surg 2007;31(4):682–9.
[3] Conte MS. The ideal small arterial substitute: a search for the Holy Grail? FASEB J 1998;12(1):43–5.
[4] Bos GW, Poot AA, Beugeling T, van Aken WG, Feijen J. Small-diameter vascular graft prostheses: current status. Arch Physiol Biochem 1998;106(2):100–15.
[5] Zilla P, Bezuidenhout D, Human P. Prosthetic vascular grafts: wrong models, wrong questions and no healing. Biomaterials 2007;28(34):5009–27.
[6] Svensson A, Nicklasson E, Harrah T, Panilaitis B, Kaplan DL, Brittberg M, et al. Bacterial cellulose as a potential scaffold for tissue engineering of cartilage. Biomaterials 2005;26(4):419–31.
[7] Oliveira RCB, Souza FC, Castro M. Avaliação da resposta tecidual quando da substituição da cartilagem do septo nasal de coelhos por manta de celulose bacteriana. Estudo experimental. Acta ORL/Técnicas em Otorrinolaringol 2007;25(4):267–77.
[8] Bodin A, Concaro S, Brittberg M, Gatenholm P. Bacterial cellulose as a potential meniscus implant. J Tissue Eng Regen Med 2007;1(5):406–8.
[9] Fontana JD, de Souza AM, Fontana CK, Torriani IL, Moreschi JC, Gallotti BJ, et al. *Acetobacter* cellulose pellicle as a temporary skin substitute. Appl Biochem Biotechnol 1990;24–25:253–64.
[10] Pippi NL, Sampaio AJSA. Estudos preliminares sobre o comportamento do Biofill na ceratoplastia lamelar em coelhos. Rev Centro Ciên Rurais 1990;20(3/4):297–302.
[11] dos Anjos B, Novaes Jr AB, Meffert R, Barboza EP. Clinical comparison of cellulose and expanded polytetrafluoroethylene membranes in the treatment of class II furcations in mandibular molars with 6-month re-entry. J Periodontol 1998;69(4):454–9.
[12] Novaes Jr AB, Novaes AB, Grissi MFM, Soares UN, Gabarra F. Gengiflex, an alkali-cellulose membrane for GTR: histologic observations. Braz Dent J 1993;4(2):65–71.
[13] Novaes Jr AB, Novaes AB. Immediate implants placed into infected sites: a clinical report. Int J Oral Maxillofac Implants 1995;10(5):609–13.
[14] Novaes Jr AB, Novaes AB. Bone formation over a TiAl6V4 (IMZ) implant placed into an extraction socket in association with membrane therapy (Gengiflex). Clin Oral Implants Res 1993;4(2):106–10.
[15] Novaes Jr AB, Novaes AB. Soft tissue management for primary closure in guided bone regeneration: surgical technique and case report. Int J Oral Maxillofac Implants 1997;12(1):84–7.
[16] Salata LA, Craig GT, Brook IM. In-vivo evaluation of a new membrane (Gengiflex®) for guided bone regeneration (GBR). J Dent Res 1995;74(3):825.
[17] Sonohara MK, Greghi SLA. Avaliação da resposta biológica a diferentes barreiras mecânicas, utilizadas na técnica de regeneração tecidual guiada (RTG). Rev Fac Odontol Bauru 1994;2(4):96–102.
[18] Klemm D, Schumann D, Udhardt U, Marsch S. Bacterial synthesized cellulose – artificial blood vessels for microsurgery. Prog Polym Sci 2001;26(9):1561–603.
[19] Brancher JA, Torres MF. Reparação microcirúrgica de nervo facial de ratos Wistar por meio de sutura–Parte II. Rev Sul-Bras Odontol 2005;2(2):34–8.
[20] Backdahl H, Helenius G, Bodin A, Nannmark U, Johansson BR, Risberg B, et al. Mechanical properties of bacterial cellulose and interactions with smooth muscle cells. Biomaterials 2006;27(9):2141–9.
[21] Backdahl H, Esguerra M, Delbro D, Risberg B, Gatenholm P. Engineering microporosity in bacterial cellulose scaffolds. J Tissue Eng Regen Med 2008;2(6):320–30.
[22] Negrão SW, Bueno RRL, Guérios EE, Ultramari FT, Faidiga AM, de Andrade PMP, et al. A eficácia do stent recoberto com celulose biosintética comparado ao stent convencional em angioplastia em coelhos. Rev Bras Cardiol Invasiva 2006;14(1):10–9.
[23] Wippermann J, Schumann D, Klemm D, Kosmehl H, Salehi-Gelani S, Wahlers T. Preliminary results of small arterial substitute performed with a new cylindrical biomaterial composed of bacterial cellulose. Eur J Vasc Endovasc Surg 2009;37(5):592–6.
[24] Helenius G, Backdahl H, Bodin A, Nannmark U, Gatenholm P, Risberg B. In vivo biocompatibility of bacterial cellulose. J Biomed Mater Res A 2006;76(2):431–8.
[25] Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin–ligand binding properties govern cell migration speed through cell–substratum adhesiveness. Nature 1997;385(6616):537–40.
[26] Salacinski HJ, Tiwari A, Hamilton G, Seifalian AM. Cellular engineering of vascular bypass grafts: role of chemical coatings for enhancing endothelial cell attachment. Med Biol Eng Comput 2001;39(6):609–18.
[27] Hoenig MR, Campbell GR, Campbell JH. Vascular grafts and the endothelium. Endothelium 2006;13(6):385–401.
[28] Kouvroukoglou S, Dee KC, Bizios R, McIntire LV, Zygourakis K. Endothelial cell migration on surfaces modified with immobilized adhesive peptides. Biomaterials 2000;21(17):1725–33.
[29] Andrade FK, Moreira SM, Domingues L, Gama FM. Improving the affinity of fibroblasts for bacterial cellulose using carbohydrate-binding modules fused to RGD. Biomed Mater Res A 2010;92(1):9–17.
[30] Costa R, Carneiro A, Rocha A, Piarraco A, Falcão M, Vasques L, et al. Bevacizumab and ranibizumab on microvascular endothelial cells. A comparative study. J Cell Biochem 2009;108(6):1410–7.
[31] Jaffe EA, Hoyer LW, Nachman RL. Synthesis of von Willebrand factor by cultured human endothelial cells. Proc Natl Acad Sci USA 1974;71(5):1906–9.
[32] Monteiro R, Calhau C, Silva AO, Pinheiro-Silva S, Guerreiro S, Gartner F, et al. Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts. J Cell Biochem 2008;104(5):1699–707.

[33] Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. Mol Cell Biol 1994;14(2):999–1008.
[34] Joshi P, Chung CY, Aukhil I, Erickson HP. Endothelial cells adhere to the RGD domain and the fibrinogen-like terminal knob of tenascin. J Cell Sci 1993;106(Pt 1):389–400.
[35] Watanabe K, Eto Y, Takano S, Nakamori S, Shibai H, Yamanaka S. A new bacterial cellulose substrate for mammalian cell culture. A new bacterial cellulose substrate. Cytotechnology 1993;13(2):107–14.
[36] Bodin A, Ahrenstedt L, Fink H, Brumer H, Risberg B, Gatenholm P. Modification of nanocellulose with a xyloglucan–RGD conjugate enhances adhesion and proliferation of endothelial cells: implications for tissue engineering. Biomacromolecules 2007;8(12):3697–704.
[37] Sanchavanakit N, Sangrungraungroj W, Kaomongkolgit R, Banaprasert T, Pavasant P, Phisalaphong M. Growth of human keratinocytes and fibroblasts on bacterial cellulose film. Biotechnol Prog 2006;22(4):1194–9.
[38] Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002;2(2):91–100.
[39] DiMilla PA, Barbee K, Lauffenburger DA. Mathematical model for the effects of adhesion and mechanics on cell migration speed. Biophys J 1991;60(1): 15–37.
[40] DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. J Cell Biol 1993;122(3):729–37.
[41] Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell 1996;84(3):359–69.
[42] Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering. J Cell Sci 2000;113(10): 1677–86.
[43] Maheshwari G, Wells A, Griffith LG, Lauffenburger DA. Biophysical integration of effects of epidermal growth factor and fibronectin on fibroblast migration. Biophys J 1999;76(5):2814–23.
[44] Wacker BK, Alford SK, Scott EA, Das Thakur M, Longmore GD, Elbert DL. Endothelial cell migration on RGD-peptide-containing PEG hydrogels in the presence of sphingosine 1-phosphate. Biophys J 2008;94(1):273–85.
[45] Cox EA, Huttenlocher A. Regulation of integrin-mediated adhesion during cell migration. Microsc Res Tech 1998;43(5):412–9.
[46] Heilshorn SC, Liu JC, Tirrell DA. Cell-binding domain context affects cell behavior on engineered proteins. Biomacromolecules 2005;6(1):318–23.
[47] Kurihara H, Nagamune T. Cell adhesion ability of artificial extracellular matrix proteins containing a long repetitive Arg–Gly–Asp sequence. J Biosci Bioeng 2005;100(1):82–7.
[48] Cavalcanti-Adam EA, Volberg T, Micoulet A, Kessler H, Geiger B, Spatz JP. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys J 2007;92(8):2964–74.
[49] Singer II, Kazazis DM. J Cell Sci 1989;93(Pt 1):147–54.
[50] Bischoff J. Cell adhesion and angiogenesis. Cell adhesion in vascular biology. J Clin Invest 1997;99(3):373–6.
[51] Xu Y, Swerlick RA, Sepp N, Bosse D, Ades EW, Lawley TJ. Characterization of expression and modulation of cell adhesion molecules on an immortalized human dermal microvascular endothelial cell line (HMEC-1). J Invest Dermatol 1994;102(6):833–7.
[52] Eliceiri BP, Cheresh DA. The role of alpha integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 1999;103(9):1227–30.