Shear stress with appropriate time-step and amplification enhances endothelial cell retention on vascular grafts

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Abstract

Endothelial cells (ECs) are sensitive to changes in shear stress. The application of shear stress to ECs has been well documented to improve cell retention when placed into a haemodynamically active environment. However, the relationship between the time-step and amplification of shear stress on EC functions remains elusive. In the present study, human umbilical cord veins endothelial cells (HUVECs) were seeded on silk fibroin nanofibrous scaffolds and were preconditioned by shear stress at different time-steps and amplifications. It is shown that gradually increasing shear stress with appropriate time-steps and amplification could improve EC retention, yielding a complete endothelial-like monolayer both in vitro and in vivo. The mechanism of this improvement is mediated, at least in part, by an upregulation of integrin β1 and focal adhesion kinase (FAK) expression, which contributed to fibronectin (FN) assembly enhancement in ECs in response to the shear stress. A modest gradual increase in shear stress was essential to allow additional time for ECs to gradually acclimatize to the changing environment, with the goal of withstanding the physiological levels of shear stress. This study recognized that the time-steps and amplifications of shear stress could regulate EC tolerance to shear stress and the anti-thrombogenic function of engineered vascular grafts via an extracellular cell matrix-specific, mechanosensitive signalling pathway and might prevent thrombus formation in vivo. Copyright © 2016 John Wiley & Sons, Ltd.

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1. Introduction

In blood vessels, endothelium is an essential modulator of vascular tone and thrombogenicity, and forms a critical barrier between the vessel wall and blood components. Endothelialization of vascular grafts before implantation has been regarded as an effective approach to prevent thrombus formation and enhance graft survival. However, endothelial cells (ECs) in the circulation are under constant blood flow-induced shear stress. Directly exposing cell-seeded vascular grafts to physiological levels of shear stress results in loss of endothelial coverage (Campbell et al., 1985; Shindo et al., 1987; Schneider et al., 1988; Hussain et al., 1991; Yazdani et al., 2014). Improving the resistance of ECs to acute shear stress would promote the long-term patency and success of tissue-engineered vascular grafts.

Many studies have demonstrated that shear stress preconditioning of ECs can improve cell retention once placed into a haemodynamically active environment (Ott et al., 1995; Meinhart et al., 2005; Inoguchi et al., 2007; Gong et al., 2014). However, the abrupt and rapid changes in shear stress applied to ECs and the effects of time-steps and amplification of shear stress on EC functions were neglected in these studies. Endothelial cells are able to sense and respond to very small changes of shear stress (Tsou et al., 2008) and abrupt, rapid changes in shear stress could affect EC functions and structure. For example, Bao et al. (1999) found the rate of change in shear stress was a potent stimulus for the expression of atherogenesis-related genes as such platelet derived growth factor (PDGF)-A and monocyte chemoattractant protein (MCP)-1. In addition, Inoguchi et al. (2007) reported that vascular endothelial (VE) cadherin, a principal cell adhesion molecule that is normally evenly distributed along the periphery of static control cells, could not be detected in human umbilical vein endothelial cells (HUVECs) after exposure to abrupt graded shear stress preconditioning conditions. Furthermore, Otte et al. (2009) found that rapid changes in shear stress induced dissociation of a Gαq/11–platelet endothelial cell adhesion molecule (PECAM)-1 complex in vitro and in vivo, which might lead to disruption of Gαq/11 trafficking and pro-inflammatory signalling. Therefore, the time-steps and amplification of shear stress may play a key role in regulating EC functions.

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Endothelial cells subjected to shear stress are able to convert mechanical stimuli into intracellular signals which affect cellular functions such as proliferation, apoptosis, migration, permeability and remodelling, as well as gene expression (Chien, 2007; Hahn and Schwartz, 2009; Collins et al., 2014). However, the mechanisms by which shear stress is transduced into cellular signalling are still unclear. Fibronectin (FN), a glycoprotein of the extracellular matrix, plays a major role in cell adhesion, growth and migration (Leiss et al., 2008). It serves as an adhesion molecule that anchors cells to extracellular matrix (ECM) mainly through integrins, which mechanically couple the actin cytoskeleton to the ECM via an elaborate adhesion complex (Friedland et al., 2009). A previous study demonstrated that the FN network became thicker, denser and highly crosslinked under physiological pulsatile flow condition and resulted in significantly improved cell retention (Gong et al., 2014). These results provided important evidence for the effects of different fluid shear stress patterns on FN assembly, which contributes to cell attachment and retention.

Located at the cell surface, integrins are possible candidates for the transduction of haemodynamic forces into biochemical signals. Integrins are heterodimeric receptors generated by selective pairing between 18 α subunits and 8 β subunits (Shyy and Chien, 2002). While many of the integrins have been shown to contribute to EC function in vitro, β1 integrin is considered to play a particularly significant role as it partners with multiple α subunits and is a highly represented receptor (Martinez-Lemus et al., 2003). Furthermore, β1 integrin on the surface of smooth muscle cells is specifically required for the assembly of ECM proteins within the vessel wall and aortic arch remodelling (Turlo et al., 2012). Clustering of β1 cytoplasmic domains of the integrin receptors activates non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and Src (Pham et al., 2000; Shyy and Chien, 2002). The composition of proteins recruited to the matrix adhesion complex specifies the biochemical signals and biophysical properties of matrix adhesions. Such a unique correlation is an important clue for the involvement of integrin β1 in mechanotransduction.

In the present study, HUVECs, one of the most commonly used model in vascular issue engineering, were seeded on silk fibroin nanofibrous scaffolds and preconditioned by shear stress using different time-steps and amplifications. The aim of the present study was to investigate the effects of the shear stress preconditioning patterns on ECs and to determine the appropriate time-steps and amplifications of shear stress required to improve EC retention on silk fibroin nanofibrous scaffolds. The retention of ECs to this substratum were examined under physiological level of shear stress in vitro and in vivo. In addition, data is presented suggesting that an increase in integrin β1 and FAK expression, which contributed to FN assembly enhancement in ECs in response to the shear stress, was responsible for the observed improved EC retention.

2. Materials and methods

2.1. Preparation of silk fibroin

Raw Bombyx mori silk fibres were supplied from Suzhou Maoda Textile Co. Ltd, China. The raw silk fibres were degummed in an aqueous solution of 0.1% (w/v) Na2CO3 at temperature between 98 and 100°C. After 30 min, the aqueous solution was refreshed; this process was repeated three times until the majority of the sericin had been removed. Afterwards, silk fibroin were dissolved in CaCl2–CH3CH2OH–H2O (mole ratio 1:2:8) at 78 ± 2°C with continuous stirring and subsequently dialysed against distilled water using a SnakeSkin Pleated Dialysis Tubing (Pierce, Rockford, USA; MWCO 3500) at room temperature. Finally, the silk fibroin solution was freeze-dried for 24 h to form silk fibroin sponges and kept in a vacuum drying desiccator for future use.

2.2. Electrospinning of silk fibroins

Silk fibroin was dissolved in hexafluoro-2-propanol (HFIP; Fluka Chemie GmbH, Munich, Germany) to generate a 10% (wt/v) solution. Electrospinning was performed with a steel capillary tube with a 1.5 mm inside diameter tip mounted on an adjustable, electrically insulated stand. The capillary tube was maintained at a high electric potential for electrospinning and was mounted in the parallel plate geometry. The capillary tube was connected to a syringe filled with the silk fibroin/HFIP solution. A constant volume flow rate of 0.8 ml/h was maintained using a syringe pump. High voltage of 10 kV was applied when the solution was drawn into fibres and these were collected on rectangular slides kept at a distance of 13 cm from the needle tip. The electrospun nanofibrous scaffolds were then treated with 100% methanol for 10 min to induce a β-sheet conformational transition, which results in insolubility in water (Liu et al., 2011).

2.3. Cell culture

The HUVECs were obtained from umbilical cord veins collected from Haidian Maternal and Child Health Hospital (Beijing, China) following a method modified from Jaffe et al. (1973). Collection and use of human umbilical cords were approved by the Ethics Committee of Haidian Maternal & Child Health Hospital. Informed consent was provided according to the Declaration of Helsinki. Briefly, umbilical cords were collected in Hanks’ balanced salt solution (HBSS) supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA) and kept at 4°C until processing. The cord was washed with HBSS. The ECs were detached from the umbilical vein lumen by short trypsinization with 1 mg/ml of collagenase (Sigma, San Francisco, CA, USA) at 37°C for 15 min. The detached cells were then collected and centrifuged at 250 g for 5 min. Supernatant was discarded and pellets were carefully resuspended in warm endothelial growth medium (EGM;
2.4. Fluid shear conditioning

The silk fibroin scaffolds fabricated on rectangular slides were sterilized by autoclave. For the shear stress loading experiment, HUVECs from passages 4–5 were cultured on sterile silk fibroin scaffolds (2.5 × 10^5 cells/scaffold). When HUVECs reached confluence, the glass slide was assembled on a parallel plate flow chamber with a flow section of 2.103 cm width, 0.05 cm height and 5.6 cm in length.

In the present study, the following shear stress preconditioning profiles were applied: (1) step flow 1 (shear stress increased from 1.2–4 dyne/cm^2) instantaneously, followed by steady shear for 8 h; shear stress was then increased stepwise in increment of 4 dyne/cm^2, followed by steady shear for 8 h, until it reached 16 dyne/cm^2); (2) step flow 2 (shear stress increased stepwise by 10% relative to the shear stress of previous hour, and sustained for 1 h, until it reached 15.7 dyne/cm^2); (3) step flow 3 (shear stress increased stepwise by 15% relative to the shear stress of previous hour and sustained for 1 h, until it reached 14.9 dyne/cm^2). Following the observation from shear conditioning pattern (1) to (3), a modified shear conditioning pattern (pattern 4) was applied: shear stress increased stepwise by 10% relative to the shear stress of the previous hour and sustained for 1 h. After the preconditioning shear stress achieved 6.8 dyne/cm^2, the sustained period increased to 2 h, until the shear stress achieved 14.9 dyne/cm^2. For convenience, the shear conditioning patterns were abbreviated as 4 dyne/cm^2/h (4 dyne/cm^2/h, 10% increase/h, 15% increase and modified, respectively, in the present study. The initial fluid shear stress used in all shear conditioning patterns was 1.2 dyne/cm^2, according to the minimum flow rate limit of the pump. The shear stress level finally achieved was near 15 dyne/cm^2, which was within the physiological range encountered in the normal arterial circulation (10–15 dyne/cm^2) (Kamiya and Bukhari, 1984; Resnick et al., 1993). Cells cultured on the scaffolds without being flow conditioned were used as static control. The gradually increased flow patterns were achieved by using a WinLJN V2.0 software for 7550 Pump Drives and Servodyne Mixers to control the speed of a peristaltic pump (Masterflex 7550; Cole-Parmer Instrument Co., Santa Clara, CA, USA), which drove the flow of media through the parallel plate flow chamber (Fig 1a–c). The flow rates to achieve desired shear stresses were calculated using the following equation (Gong et al., 2014):

\[ Q = \frac{\tau \rho h^2}{6 \mu} \]

where \( Q \) is the volumetric flow rate, \( \tau \) is the shear stress to which the HUVEC were exposed, \( \mu \) is the dynamic viscosity of the culture medium, which is 0.7544 cp in the present study, and \( \omega \) and \( h \) are flow channel width and height, respectively. The culture medium was the same as that mentioned in the cell culture section. Cells on silk fibroin scaffolds were assessed every 8 h and at the end of each shear conditioning process according to the time-steps and amplifications of fluid shear stress, respectively.

2.5. Cell labelling and detection

Fluorescein diacetate (FDA) molecule probe is well recognized as a viable cell marker. Cells on silk fibroin scaffolds were rinsed twice with sterile HBSS, incubated in 10 ml of FDA (Sangon Biotech, Shanghai, China) working solution (10 μg/ml) at 37°C for 15 min, and then washed with HBSS twice. Subsequently, cells were observed and photos were taken by an inverted fluorescence microscope (IX71; Olympus Inc., Tokyo, Japan).

2.6. Fluorescent staining

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) and then blocked in 1% bovine serum albumin (BSA). Primary antibodies to Integrin β1 (1:200; Biologieg Biotech, USA), FAK (1:100; Biossen Biotech, Beijing, China), FN (1:100; Boster Biotech, Wuhan, China) and CD41 (1:100, Biossen Biotech, Beijing, China) were incubated with the cells at 4°C overnight. After washing with PBS, tetramethylrhodamine (TRITC) conjugated antibodies (Zhongshan, Beijing, China)–fluorescent staining was visualized and photos were taken under a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany) using a × 63 oil immersion objective lens. Fluorescence intensity was determined using Leica software. Cell area was determined by manual delineation of raw fluorescence images. Integrin β1 and FAK expression levels were expressed as average intensities in arbitrary fluorescence units (a.u.). At least three distinct positions of a sample were photographed and analysed.

2.7. Cell adhesion assay

Cell-adhesion assays were performed in 96-well plates, in triplicate, with a dye-staining method, as described previously (Humphries, 2001). Unless otherwise stated HUVECs were pretreated under different conditions, collected and plated for adhesion assay at 4 × 10^5 cells on a 96-well plate.
coated with FN (5 μg/cm²). Cells were incubated at 37°C for 40 min, fixed in 4% formaldehyde, washed in PBS, and stained with 1% crystal violet for 1 h. Then, 10% acetic acid was added to the dried plate, and optical density (OD) at 570 nm was measured to detect cell adhesion.

To identify FN involved in EC adhesion, the 96-well plates were coated with FN (0–50 μg/ml; Merck, Nottingham, England), blocked with the blocking buffer (PBS with 1% BSA) for 0.5 h and then the cell attachment was assessed as described above.

To identify integrin β1 involved in EC adhesion, HUVECs were preincubated with 10 μg/ml function-activating antibodies (mouse anti human integrin β1 clones, TS2/16; Biolegend, San Diego, CA, USA) for 20 min, or with 10 μg/ml of rabbit anti-human integrin β5 antibody (Immunoway, Newark, DE, USA) to identify non-specific interactions. To confirm the results of the integrin involvement assay, cells were preincubated with RGDS blocking peptides (0–2 mM; Calbiochem, La Jolla, CA, USA) for 20 min before plating. Poly-L-lysine-coated (50 μg/ml; Sigma, St. Louis, MO, USA) wells were used to identify non-specific interactions.

To identify shear conditioning involved in EC adhesion by integrin β1, HUVECs on silk fibroin scaffolds were precultured under shear conditioning pattern 2 or static conditions for 24 h, harvested using trypsin, plated in

Figure 1. Effects of time steps and amplifications of fluid shear stress in shear conditioning on the retention of human umbilical vein endothelial cells (HUVECs) cultured on silk fibroin nanofibrous scaffolds. The initial fluid shear stress, which was limited by the minimum flow rate of the peristaltic pump, was 1.2 dyne/cm² in all flow conditioning patterns. (a) Schematic illustration of the bioreactor system for fluid shear conditioning (not drawn to scale). (b) Photograph of the bioreactor system set-up. (c) Photograph of the parallel plate flow chamber. (d) An increase of 4 dyne/cm² every 8 h to 16 dyne/cm² over 40 h, except that the first increase of shear stress was from 1.2 dyne/cm² to 4 dyne/cm². Cells were significantly lost at 8 dyne/cm² (24 h) and completely lost at 12 dyne/cm² (32 h). (e) An increase of 10% from the shear stresses of the previous hour, with a goal of 15.7 dyne/cm² after 28 h. The modest increase in shear stress enhances cell retention at 15.7 dyne/cm² (28 h). (f) An increase of 15% from the shear stresses of previous hour, with a goal of 14.9 dyne/cm² after 19 h. Cells were nearly completely dislodged at 9.8 dyne/cm² (16 h) and completely dislodged at 14.7 dyne/cm² (19 h). (g) Confluent HUVECs of the static culture control without flow conditioning. (h) Poor retention of endothelial cells (ECs) when the static culture control was exposed to a physiological magnitude of shear stress (12 dyne/cm²) for 8 h. (i) Quantification of the fluorescence images by image analysis showed significantly improved cell retention of HUVECs of the 10% increase/h group. Data are mean ± SD, n = 3; *significant difference between the 10% increase/h, 15% increase/h and 4 dyne/8 h groups at p < 0.05. Bar, 100 μm.
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the experimental wells at a concentration of $4 \times 10^5$ cells/well, and then allowed to attach for 40 min at 37°C. To confirm the results that shear conditioning involved in EC adhesion by integrin β1, cells were preincubated with RGDS blocking peptides (1 mM; Calbiochem) for 20 min before plating.

To identify FAK involved in EC adhesion, cells were preincubated with FAK inhibitor 14[1,2,4,5-benzene-tetraamine tetrahydrochloride (Y15)] (0–50 μM; Tocris, Ellisville, MO, USA) to inhibit FAK activity and expression for 20 min before plating.

2.8. Cell retention evaluation

Human umbilical vein endothelial cells were cultured on glass slides coated with FN (5 µg/cm²) to confluence, exposed to 10 min continuous steady laminar shear stress (LSS) at 15, 30 and 60 dyne/cm² which represent the normal, high and very high levels, respectively, of shear stress in vivo (Feugier et al., 2005). Subsequently, HUVECs remained on the glass slides were stained with FDA and visualized by an inverted fluorescence microscope (IX71; Olympus Inc.). Micrographs of cell-covered areas were quantified with a self-written image processing program (mat-lab2010b, Natick, MA, USA). The differences in cell numbers before and after LSS exposure were calculated. The quantity of HUVEC attachment was measured by the extent of resistance to the shear stress. The HUVECs cultured on uncoated glass slides were used as a control. At least three distinct positions of a sample were photographed and calculated.

Human umbilical vein endothelial cells on silk fibroin scaffolds were incubated under the modified shear conditioning pattern (pattern 4) for 32 h to make the shear stress reach 12 dyne/cm², and then the cells were exposed to LSS of 12 dyne/cm² for 24 h, which represents the typical mean shear stress at the femoral artery. Cells cultured without shear conditioning were exposed to LSS of 12 dyne/cm² for 24 h as a control. The HUVECs remaining on the silk fibroin scaffolds were stained, visualized and quantified by the methods described above.

2.9. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay

The HUVECs on silk fibroin scaffolds were incubated under the modified shear conditioning pattern (pattern 4) for 32 h and then exposed to LSS of 12 dyne/cm² for 24 h. Cell apoptosis of HUVECs cultured on silk fibroin scaffolds before and after exposure to LSS was quantified with a TUNEL assay. This assay was performed using a commercial kit (In Situ Cell Death Detection Kit; Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. In brief, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS and then incubated with TUNEL reaction mixture at 37°C for 60 min in a dark humidified chamber. Stained cells were visualized and photographed by an inverted fluorescence microscope (IX71; Olympus Inc.). Nuclei with clear green staining were regarded as positive. Nuclei with blue staining represented the total cells. For each sample, at least three randomly selected fields at ×200 magnifications were evaluated. Cells cultured without shear conditioning were exposed to LSS of 12 dyne/cm² for 24 h as a control.

2.10. Platelet adhesion experiments

Human platelets were used to evaluate the capability of the regenerated endothelium to prevent thrombosis. Fresh human venous blood used in the experiments was obtained from the Beijing Red Cross Blood Centre, China. Whole human blood was collected with anticoagulant. Blood samples were centrifuged at 280 g (10 min) at room temperature and the platelet-rich plasma (PRP) was collected for experiments (Hashi et al., 2007). Fresh PRP samples were used in all the studies. The fresh PRP was diluted with Tyrode’s buffer to a concentration of $\sim 1 \times 10^9$ platelets/ml and then incubated at 37°C for 30 min with HUVECs that had been cultured on silk fibroin scaffolds under modified shear conditioning for 32 h and then exposed to LSS of 12 dyne/cm² for 24 h. Cells cultured without shear conditioning were exposed to LSS of 12 dyne/cm² for 24 h as a control. Subsequently, samples were washed in PBS three times to remove non-adherent platelets and fixed in 4% paraformaldehyde. Adherent platelets were investigated by immunofluorescence. Adherent platelets were stained by rabbit anti-human CD41 (1:100; Biossen Biotech) and goat anti-rabbit IgG TRITC conjugate while the HUVECs were stained by mouse anti-human β-catenin (1:100; Boster Biotech) and goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Zhongshan). Nuclei were labelled with DAPI (Sigma).

2.11. Statistical analysis

Each experiment was repeated independently at least three times. All data is expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. A value of p < 0.05 was considered statistically significant.

3. Results

3.1. Effect of time-steps and amplifications of fluid shear stress in shear conditioning on HUVEC retention

The aim of the present study was to evaluate the effects of time-steps and amplifications of fluid shear stress on the retention of HUVECs cultured on silk fibroin nanofibrous scaffolds. The HUVECs cultured on scaffolds without flow conditioning were used as static control. As shown in Figure 1, the confluent cell monolayer of the static control was completely dislodged after an 8-h exposure to a physiological level of shear stress (12 dyne/cm², Figure 1g,h), which suggested the need for improvement of the protocol.
for endothelialization of the vascular grafts. For the 4 dyne/8 h group, HUVECs began to detach from the scaffold surface when the fluid shear stress reached 4 dyne/cm² after 16 h, and completely dislodge at 12 dyne/cm² after 32 h (Figure 1d). For the 10% increase/h group, a confluent monolayer of cells was maintained until the fluid shear stress reached 5 dyne/cm² over 16 h. When the fluid shear stress reached 15.7 dyne/cm² after 28 h, ~5.49 ± 0.99% of cells were still retained on the surface (Figure 1e,i). For the 15% increase/h group, HUVECs began to detach at 3.2 dyne/cm² after 8 h and almost completely dislodged at 9.8 dyne/cm² after 16 h (Figure 1f). Quantification of these results by image analysis indicated that cell retentions of the three sample groups under physiological ranges of fluid shear stress were 45.82 ± 5.98% for the 10% increase/h group at 10.7 dyne/cm² over 24 h, 38.58 ± 7.55% for the 4 dyne/8 h group at 8 dyne/cm² over 24 h and 2.04 ± 0.22% for the 15% increase/h group at 9.8 dyne/cm² over 16 h (Figure 1i). Compared with the abrupt increase group with large amplification (4 dyne/8 h and 15% increase/h), the modest increase group with small amplification (10% increase/h) demonstrated a significant improvement in cell retention.

3.2. Effect of time-steps and amplifications of fluid shear stress in shear conditioning on FN assembly

The possible mechanism for improved attachment of HUVECs by shear conditioning was considered next. Previous studies demonstrated that flow patterns had strong

![Image](99x134 to 497x579)
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effects on FN assembly, which played a critical role in cell attachment (Gong et al., 2014). The present study investigated whether the fluid shear stress with different time-steps and amplifications had effects on FN assembly. Cell attachment assays showed that FN supported HUVEC attachment in a dose-dependent manner (Figure 2a). The retention of ECs on FN substratum after exposure to acute shear stress was evaluated next. The cell retention on the FN substratum was significantly higher at all shear stress levels (15, 30 and 60 dyne/cm²) than that on the glass substratum without FN coating (Figure 2b, p < 0.05), which demonstrated that FN improved cell retention under shear stress.

Immunostaining was conducted to assess changes in FN assembly (Figure 2c–n). No notable changes in FN assembly were observed in the cells of the 4 dyne/8 h or 10% increase/h groups compared with the static control after 8 h of flow exposure (Figure 2c–n). Thick and dense FN network was observed in the cells of the 10% increase/h group after 16 h of flow exposure (Figure 2h). However, for the cells in the 4 dyne/8 h or 10% increase/h group, much sparser FN fibrils networks were observed after 24 h of flow exposure (Figure 2d–i). Cells in the 4 dyne/8 h group had a more patchy FN fibril network appearance than that of the 10% increase/h group, which was consistent with lower cell retention, according to the results shown in Figure 1d. Furthermore, granular FN and short FN fibrils were observed in the cells in the 10% increase/h group after 24 h of flow exposure (Figure 2i,h), indicating that abrupt change in fluid shear stress (from 5 dyne/cm² to 10.7 dyne/cm²) affected FN assembly.

Figure 3. The time-steps and amplifications of fluid shear stress in shear conditioning affect expression of integrin β1, which is essential to endothelial cell (EC) attachment and retention. (a) Cell attachment improved by shear conditioning is dependent on integrin β1. Human umbilical vein endothelial cells (HUVECs) were treated with function-activating antibody against integrin β1, RGDS blocking peptide or antibody against integrin β5, or were pre-flow conditioned under the 10% increase/h pattern for 24 h and were detached and incubated with/without RGDS blocking peptide. The pretreated HUVECs were then replated on cell culture plates. Data are mean ± SD, n = 3; significant difference between the treated group and the untreated control group at p < 0.01. (b) FGDS peptides inhibit cell attachment on fibronectin (FN) (5 μg/cm²) substrata in a dose-dependent manner. No inhibition was observed on poly-L-lysine (50 μg/ml) substrata. (c,d) Integrin β1 enhances FN assembly. The HUVECs were incubated with (d)/without (c) Integrin β1-activating antibody (10 μg/ml) for 16 h. (e–o) Effects of the time-steps and amplifications of fluid shear stress in shear conditioning on integrin β1 expression. The HUVECs cultured on silk fibroin scaffolds were assessed every 8 h and at the end of each flow conditioning process (40 h for the 4 dyne/8 h group, 28 h for the 10% increase/h group and 19 h for 15% increase/h group). (p) Integrin β1 expression of the static control. A modest increase in fluid shear stress enhances integrin β1 expression of HUVECs. Bars, 25 μm. (q–s) Quantification of immunofluorescent intensity of Integrin β1 under different flow conditioning patterns: 4 dyne/8 h group (q), 10% increase group (r) and 15% increase group (s). Data are mean ± SD, n = 3; * p < 0.01 compared with immunofluorescent intensity value of 0 h 0 dyne.
organization. This result was consistent with the cell dislodgement observed at 24 h in Figure 1e. Similarly, a granular appearance and short fibrils were observed in cells of the 15% increase/h group after 8 h of flow exposure (Figure 2k,n), and no FN was observed after 16 h of flow exposure (Figure 2i). Together, these data suggest that the shear conditioning patterns affected the dynamic organization of the FN network and contributed to cell attachment and retention. A modest increase in fluid shear stress enhanced FN assembly, while dramatic changes in fluid shear stress led to the disruption of FN network.

3.3. Effect of time-steps and amplifications of fluid shear stress in shear conditioning on integrin β1 expression

As integrin β1 plays a critical role in FN assembly and cell attachment (Wierzbiacka-Patynowski and Schwarzbauer, 2003; Turlo et al., 2012), it was decided to examine whether shear conditioning might have effects on integrin β1 expression. The HUVECs cultured on silk fibroin nano-fibrous scaffolds were flow conditioned (10% increase/h) for 24 h and were then plated onto 96-well plate precoated with FN. When compared with the static cultured HUVECs (control), the flow conditioned cells demonstrated a 55.04 ± 6.45% increase in attachment to FN. Addition of RGDS blocking peptides significantly reduced EC attachment to FN substratum either under static control culture condition or under shear conditioning (Figure 3a). The inhibition was dose-dependent, with half-maximal response achieved at 0.5 mM RGDS. The addition of RGDS peptides did not significantly inhibit EC attachment to poly-l-lysine substratum (Figure 3b). Moreover, on FN substratum, function-activating anti-β1 (clones TS2/16) antibody significantly increased EC attachment by 32.03 ± 2.44% (p < 0.05) compared with the untreated control. Incubation with anti-β5 antibody resulted in no significant difference in cell attachment (Figure 3a). Consistently, function-activating anti-β1 (clones TS2/16, 10 μg/ml, 16 h treated) antibody significantly enhanced FN assembly compared with the untreated control (Figure 3c,d). These results indicate that integrin β1 can improve FN assembly and cell attachment.

Immunofluorescence imaging of integrin β1 in HUVECs under shear conditioning was conducted to assess the changes in expression (Figure 3e–p). Staining was quantified by measuring fluorescence intensity (Figure 3q–s). Integrin β1 expression for the 10% increase/h group was slightly increased at the first 8 h, and then was significantly increased after 16 h of shear conditioning (Figure 3i–l,p,r, p < 0.01) when compared with the static control. For the 4 dyne/8 h group, a slight increase of integrin β1 expression was observed at the first 8 h of exposure, and then the integrin β1 staining was significantly decreased after prolonged shear conditioning (Figure 3e–h,p,q, p < 0.01). Similar results were observed in the 15% increase/h group (Figure 3m–o,p,s, p < 0.01), demonstrating that dramatic changes in fluid shear stress leads to inhibition of integrin β1 expression.

3.4. Effect of time-steps and amplifications of fluid shear stress in shear conditioning on FAK expression

Focal adhesion kinase is an important integrin β1 downstream signalling molecule and is essential for EC attachment and retention (Shy and Chien, 2002; Singh et al., 2010), thus, the effect of time-steps and amplifications of fluid shear stress in shear conditioning on FAK expression was investigated. Cell attachment assays demonstrated that FAK inhibitor 14 inhibited HUVEC attachment to FN substratum in a dose-dependent manner, with maximal inhibition detected at the 20 μM dose (Figure 4a). The HUVECs were next treated with 10 μM inhibitor 14 for 0, 1, 2 and 4 h. Attachment of HUVEC to FN substratum was inhibited in a time-dependent manner (Figure 4b). To assess changes in cell morphology, HUVECs were cultured on silk fibroin nano-fibrous scaffolds and treated with 10 μM inhibitor 14 for 2 h and 4 h. The morphology analysis with inhibitor 14 showed significant cell detachment compared with the untreated control (Figure 4c–e). The cellular detachment was time-dependent, which was consistent with the results shown in Figure 4b. The inhibitor 14-treated HUVECs became completely dislodged from silk fibroin nano-fibrous scaffolds after being flow conditioned for 2 h and 4 h (Figure 4f–g).

The HUVEC FN matrix in the 2 h-inhibited group had a granular appearance and displayed a poorly organized array of fibrils compared with the thin and long FN fibrils in the uninhibited control group (Figure 4h,i). More sparse and poorly organized FN fibrils were observed for the 4 h-inhibited group (Figure 4j), which demonstrated the important role of FAK in FN fibril organization. The FN matrix in the 2 h-inhibited HUVEC group was significantly decreased after being shear conditioned for 2 h, and completely disappeared after 4 h shear conditioning (Figure 4k,l). These results were consistent with cell dislodgement shown in Figure 4f–g, and demonstrated the importance of FN assembly in cell resistance to shear stress.

The effect of time-steps and amplifications of fluid shear stress on FAK expression was then evaluated. Figure 4m–x shows the immunofluorescence images of FAK expression in HUVECs under shear conditioning. Staining was quantified by measuring the intensity of fluorescence (Figure 4a–c). A significant increase in FAK expressions was observed for the 4 dyne/8 h group and the 10% increase/h group after 16- and 24-h shear conditioning when compared with the static control group (Figure 4n,s,a,b,i, p < 0.01). The FAK expression peak levels in the 10% increase group level (49.56 ± 5.27, 24 h) was higher than that of the 4 dyne/8 h group (37.61 ± 5.25, 16 h). Based on these observations, the changes in integrin β1 observed after 16 h of shear conditioning (Figure 3i) precede the changes in FAK.

3.5. The modified shear conditioning pattern enhances cell retention, integrin β1 expression, FAK expression and FN assembly

A modest increase in fluid shear stress was shown to enhance cell retention by improving the integrin β1 expression,
FAK expression and FN assembly (Figures 2–4). However, the cell retention rate decreased from nearly 100% at 16 h to 45.82% at 24 h for the 10% increase/h group because of dramatic changes in fluid shear stress (from 5 to 10.7 dyne/cm²). Thus, the time-step was modified to slow down the shear stress increase for HUVECs to allow them to acclimatize to their changing microenvironment within the bioreactor. For the new conditioning profile, the shear stress...
increased stepwise by 10% relative to the shear stress of the previous hour and was sustained for 1 h. After the preconditioning shear stress achieved 6.8 dyne/cm², the sustained period increased to 2 h until the shear stress achieved 14.9 dyne/cm².

The results of this modified protocol are illustrated in Figure 5. A complete monolayer of HUVECs was observed over the entire luminal surface at 11.8 dyne/cm² over 32 h. Most cells remained until the fluid shear stress reached 15.7 dyne/cm² over 38 h (Figure 5a).

Quantification of the results at these time-points by image analysis indicated that the cell retention rates were 99.31 ± 4.97% at 32 h and 71.13 ± 12.82% at 38 h compared with the static control (Figure 5b), demonstrating a significant improvement of cell retention under physiological level of fluid shear stress. The integrin β1 expression was significantly increased at 16 h compared with the static control and maintained a high level of expression throughout the subsequent conditioning process (Figure 5c–h, p < 0.01). Expression of FAK was significantly increased at 24 h and was maintained at a high level throughout the subsequent conditioning process (Figure 5i–n, p < 0.01) compared with the static control. The FAK expression peak was later (24 h) than the appearance of integrin β1 expression peak (16 h) (Figure 5u–v). The fluorescent images consistently showed that FN continued to be assembled into the network matrix during the flow conditioned process, leading to a dense and highly crosslinked FN fibril network surrounding HUVECs in the monolayer by 32 h (Figure 5o–t) compared with the static control.

3.6. The modified shear conditioning pattern enhances EC retention and anti-platelet adhesion in vitro

To evaluate whether the flow conditioned ECs can resist long-term exposure to a physiological magnitude of fluid shear stress, HUVECs cultured on silk fibroin nanofibrous scaffolds were preconditioned according to the modified protocol for 32 h and were exposed to 12 dyne/cm² fluid shear stress, which is a typical mean shear stress at the femoral artery. As shown in Figure 6a, a confluent monolayer of ECs was retained after exposure to fluid shear stress for 24 h. Apoptotic cells were assessed by TUNEL assay. No cell apoptosis was found for the preconditioned group (Figure 6b,c). In contrast, no cells were found on the surface of the static control (Figure 6e–g).

Platelet adhesion is thought to be a major mechanism by which biomaterial thrombogenicity is transduced (Roald et al., 1994; Hashi et al., 2007). Thus, in vitro experiments were carried out to determine platelet adhesion.
on the scaffold surfaces after exposure to 12 dyne/cm² fluid shear stress for 24 h. The fluorescence images revealed very little platelet adhesion on the surfaces of flow conditioned samples (Figure 6d). In contrast, there were significant amounts of platelets adhering to the scaffold surfaces of the static control (Figure 6h). The results suggest that the flow conditioned samples could resist the physiological fluid shear stress while remaining anti-thrombogenic properties.

4. Discussion

Several previous studies have suggested that shear stress preconditioning of EC-seeded vascular grafts could improve cell retention and functions (Ott et al., 1995; Dardik et al., 1999; Meinhart et al., 2005). However, ECs are very sensitive to the changes in shear stress, the time-steps and amplifications of shear stress, which are all important but neglected factors in regulating the EC function. It has been demonstrated that abrupt and rapid changes in shear stress might affect the function and structure of ECs. The present study indicates that gradually increased shear stress with appropriate time-steps and amplification can improve EC retention, yielding a complete endothelial-like monolayer both in vitro and in vivo. The mechanism of this improvement is shown to be mediated, at least in part, by upregulation of the FN assembly, integrin β1 and FAK expression in response to the mechanotransduction of fluid-induced shear stress.

To develop a complete EC monolayer with high shear-stress resistance characteristics, HUVECs were cultured on silk fibroin nanofibrous scaffolds and exposed to shear stress with different time-steps and amplifications. The results showed that cell retentions under fluid shear stress within physiological ranges were 45.82 ± 5.98% for the 10% increase/h group at 10.7 dyne/cm² over 24 h, 38.58 ± 7.55% for the 4 dyne/8 h group at 8 dyne/cm² over 24 h and 2.04 ± 0.22% for the 15% increase/h group at 9.8 dyne/cm² over 16 h (Figure 1f). Compared with the abrupt increase groups with large amplification (4 dyne/8 h and 15% increase/h), the modest increase group with small amplification (10% increase/h) demonstrated a significant improvement in cell retention. In addition, the cell retention rates decreased from nearly 100% at 16 h to 45.82% at 24 h for the 10% increase group because of the dramatic changes in fluid shear stress (from 5 dyne/cm² to 10.7 dyne/cm²). To optimize the technique of shear conditioning, the time steps were changed from 10% increase/h to 10 increase% every 2 h. In comparison with all other groups, the modified protocol resulted in 99.31 ± 4.97% cell retention at 11.8 dyne/cm² over 32 h, with a nearly complete monolayer of HUVECs covering the entire luminal surface (Figure 5b). This modified time-step allowed more time for ECs to gradually acclimatize to the changing environment with the goal of sustaining physiological levels of shear stress, which resulted in excellent EC retention on the silk fibroin scaffolds. Moreover, after 24 h of implantation, the vascular grafts showed excellent patency with a nearly confluent EC monolayer along the entire length of the graft.

The mechanism of how the time-steps and amplifications of shear stress influence EC attachment and retention was evaluated in the present study. The effects of the time-steps and amplifications of shear stress on FN matrix formation and distribution were investigated first, as FN is an ECM protein that has been found to be important in cell attachment processes. In addition, FN assembly plays a central role in blood vessel organization by regulating both ECM composition and the deposition of other ECM molecules, including collagen types IV and...
laminin (Ruoslahti, 1988; Hynes and Zhao, 2000; Kim et al., 2000). As shown in Figure 2, the time-steps and amplifications of shear stress affected the dynamic organization of the FN network. A modest increase in fluid shear stress enhanced FN assembly, while dramatic changes in fluid shear stress led to the disruption of FN network. Mott and Helmke’s (2007) report indicated that the onset of 15 dyne/cm² of shear stress decreased the displacement of focal adhesion and FN fibrils. Previous studies also showed that FN could be structurally rearranged by ECs in response to different flow patterns (Gong et al., 2014). In the present study, ECs exposed to the modified shear preconditioning pattern revealed an observable increase in FN fibrils and denser FN matrix, measured through increased immunofluorescent intensity, which demonstrated the importance of time-steps and amplifications of shear stress on FN matrix production and distribution.

The results of the present study further indicate that activation of integrin β1 could improve the FN assembly and enhance cell attachment to FN (Figure 3c,d). Integrin β1 plays a particularly significant role in mediating FN assembly and cell–matrix adhesion (Wu et al., 1995; Lei et al., 2008; Malan et al., 2010; Zou et al., 2012). Following binding to FN via the RGD region, integrin β1 clusters form focal adhesion complexes that are responsible for strong interactions between cells and their surrounding matrix. The clustering of β1 integrin leads to activation of non-receptor tyrosine kinases such as FAK and c-Src, Proto-oncogene tyrosine-protein kinase, and initiates responses within the cell that make it possible for the cell to organize a fibrillar FN matrix (Wu et al., 1995; Zou et al., 2012). Truncation of the β1 cytoplasmic domain has been shown to block FN matrix assembly by severing the link between ligand-occupied integrins and the cytoskeleton (Wu et al., 1995). Elloumi-Hannachi et al. (2014) have consistently found that deletion of β1 significantly reduced integrin binding to fibronectin and lowered adhesion strength, while expression of human β1 integrin restored cell adhesive functions.

Integrin-elicited signalling events are commonly investigated by allowing cells to attach to ECM proteins or by treating cells with monoclonal antibodies against integrins (Wu et al., 1995; Urbich et al., 2000; Petzold et al., 2013). However, many of the signalling pathways activated by these stimuli are also activated by shear stress, which suggests that integrins are involved in mechanotransduction in ECs (Shy and Chien, 2002; Chien, 2007; Hahn and Schwartz, 2009; Steward et al., 2011). In the present study, HUVECs that were pre-exposed to shear conditioning demonstrated a significantly increased attachment to FN (155.04 ± 6.45% of static controls). This improvement of cell–matrix adhesion can be inhibited by RGDS blocking peptides, indicating that integrin β1 is involved in the shear conditioning-induced cell–matrix adhesion. The results are in line with Urbich et al.’s (2000) work, which showed that shear stress enhanced EC adhesion to FN via upregulation of integrin β1. Moreover, although integrin β1 expression in all the preconditioning groups increased compared with the static control (Figure 3q–s), significant increase was observed only in the 10% increase group and the modified group. The results demonstrate that time-step and amplification of shear stress has obvious effects on the regulation of integrin β1 expression and FN assembly (Figures 2h, 5t). The gradual introduction of shear force promoted ECs to adapt to their microenvironment through modulation of integrin β1.

Focal adhesion kinase is an important downstream integrin β1 signalling molecule and is a structural enzymatic component that co-localizes with integrin in focal adhesions. These proteins connect the extracellular filamentous meshwork to the intracellular cytoskeleton conferring on them the status of an ideal checkpoint capable of controlling or mediating bidirectional mechanical transduction through ECs. Several previous studies have shown that FAK is necessary for integrin-mediated FN assembly (Kim et al., 2000; Ilić et al., 2004; Singh et al., 2010; Steward et al., 2011; Kim and Wirtz, 2013). For example, Ilić et al. (2004) found that the loss of FAK primarily influenced matrix organization or deposition rather than FN gene function. Fibronectin staining in permeabilized FAK−/− cells had significantly shorter FN fibrils compared with the FAK+/+ controls (Ilić et al., 2004). The results of the present study are consistent with these findings. After HUVECs were treated with FAK inhibitor 14, FN assembly was blocked and the shear stress tolerance of HUVECs was significantly decreased (Figure 4a–l). Analysis of shear stress-induced and cyclic stretch-induced signalling suggested that dynamic interactions between integrin molecules present in focal adhesion complexes and FAK triggered the remodelling of cell contacts and cytoskeleton as well as induced other intracellular signals leading to integrated EC response to mechanical stimuli (Shy and Chien, 2002; Davies, 2009; Petridou and Skourides, 2014). The modest loading method of shear stress used in the present study upregulated FAK expression (Figure 5I) and enhanced FN assembly (Figure 5t), while dramatic changes in fluid shear stress led to no significant changes in FAK immunostaining and very short FN fibrils compared with the static control (Figures 2i and 4s). These findings confirmed the important role of time-steps and amplifications of shear stress on FAK-mediated FN matrix assembly.

Laminar shear stress can inhibit apoptosis of ECs in response to various stimuli, demonstrating the potent atheroprotective effects of shear stress to preserve the integrity of the endothelium. The survival of ECs is critical for the maintenance of blood vessel integrity and angiogenesis. A number of studies have addressed the possibility that shear stress-induced upregulation of integrins enhances stimulation of integrin signalling and thereby promotes EC survival (Dimmeler et al., 1996, 1998; Urbich et al., 2000). Moreover, deletion of FAK or displacement of FAK from focal adhesions may affect cytoskeletal organization and cell migration, as well as cell survival and cell cycle progression (Schaller, 2001; Sechler et al., 2001). These conclusions are also supported by the
findings of the present study that the gradual increase of shear stress enhances integrin and FAK expression, FN assembly and EC survival. In addition, a study performed by Wary et al. (1996) found that the adhesion of HUVECs to FN promoted EC survival via activation of the p52Shc adapter protein, which in turn recruited the Grb2-mSOS complex to the membrane and thereby activated the MAP kinase pathway.

5. Conclusions

Based on the above-mentioned results, it is concluded that a modest gradual increase in shear stress is essential to allow additional time for ECs to gradually acclimatize to the changing environment with the goal of withstanding the physiological levels of shear stress. Gradually increased shear stress with appropriate time-step and amplification improved EC retention, yielding a complete endothelial-like monolayer both in vitro and in vivo. The results further indicated that the increase in integrin β1 and FAK expression, which contributed to enhancement of FN assembly in ECs in response to the shear stress, was responsible for the observed improved EC retention. The time-steps and amplifications of shear stress can regulate EC tolerance to shear stress and anti-thrombogenicity of engineered vascular grafts via an ECM-specific, mechanosensitive signalling pathway, in which integrin β1, FAK and FN play a major role. These findings may contribute to the functional integrity of the EC monolayer and thereby inhibit damage to the arterial wall, which is a key event for thrombogenicity or intimal hyperplasia.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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