J. Cell. Mol. Med. Vol 16, No 9, 2012 pp. 2117-2126

A fibronectin-fibrinogen-tropoelastin coating reduces smooth muscle cell growth but improves endothelial cell function

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Received: August 23, 2011; Accepted: December 16, 2011

Abstract

Reendothelialization of the stent surface after percutaneous coronary intervention (PCI) is known to be an important determinant of clinical outcome. We compared the effects of biological stent coatings, fibronectin, fibrinogen and tropoelastin, on human umbilical vein endothelial cell (HUVEC) and vascular smooth muscle cell (VSMC) characteristics. Umbilical cord arterial segments were cultured on coated surfaces and VSMC outgrowth (indicating proliferation and migration) was measured after 12 days. mRNA was isolated from HUVEC and VSMC cultured on these coatings and gene expression was profiled by QPCR. Procoagulant properties of HUVEC were determined by an indirect chromogenic assay which detects tissue factor activity. The varying stent coatings influence VSMC outgrowth: 31.2 ± 4.0 mm^2 on fibronectin, 1.6 ± 0.3 mm^2 on tropoelastin and 8.1 ± 1.5 mm^2 on a mixture of fibronectin/fibrinogen/tropoelastin, although HUVEC migration remains unaffected. Culturing HUVEC on tropoelastin induces increased expression of VCAM-1 (13.1 ± 4.4 pg/ml), ICAM-1 (5.1 ± 1.3 pg/ml) and IL-8 (11.6 ± 3.1 pg/ml) compared to fibronectin (0.7 ± 0.2, 0.8 ± 0.2, 2.3 ± 0.5 pg/ml, respectively), although expression levels on fibronectin/fibrinogen/tropoelastin remain unaltered. No significant differences in VCAM-1, ICAM-1 and IL-8 mRNA expression are found in VSMC. Finally, HUVEC cultured on tropoelastin display a fivefold increased tissue factor activity (511.6 ± 26.7%), compared to cells cultured on fibronectin (100 ± 3.9%) or fibronectin/fibrinogen/tropoelastin (76.3 ± 25.0%). These results indicate that tropoelastin inhibits VSMC migration but leads to increased inflammatory and procoagulant markers on endothelial cells. Fibronectin/fibrinogen/tropoelastin inhibits VSMCs while compensating the inflammatory and procoagulant effects. These data suggest that coating a mixture of fibronectin/fibrinogen/tropoelastin on a stent may promote reendothelialization, while keeping unfavourable processes such as restenosis and procoagulant activity limited.

Keywords: stent • endothelial cells • restenosis • inflammation • platelets

Introduction

Stent implantation after PCI is a widely applied method to dilate stenotic coronary arteries. Major complications of stent placement are restenosis and in-stent thrombosis. Bare-metal stents provoke a cascade of unfavourable inflammatory responses, starting with cytokine release leading to the infiltration of inflammatory cells and to the activation of VSMCs in the tunica media [1, 2], eventually leading to restenosis of the stented segment. Contractile VSMCs are present in all normal healthy arteries to sustain vascular tone and resistance. Upon injury, the cells differentiate into synthetic VSMCs and gain the ability to proliferate and migrate [3]. This phenotype switch leads to decreased expression of SM α-actin mRNA, increased growth factor expression and deposition of extracellular matrix [3–5].

Drug-eluting stents (DES) significantly reduce the incidence of in-stent restenosis by inhibiting proliferation of VSMC and endothelial cells (ECs). Proliferation of ECs, however, is necessary for reendothelialization of the stent surface and a delay in this process can result in late stent thrombosis [6]. ECs proliferate and migrate from the injured vessel wall onto the stent struts to cover the metal surface. Furthermore, previous studies have shown the capacity of circulating endothelial progenitor cells to home to exposed subendothelium and thereby promoting reendothelialization [7, 8]. Coatings

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doi: 10.1111/j.1582-4934.2011.01519.x

promoting the reendothelialization have been studied extensively. These studies focus on the use of antibodies or peptides to capture endothelial progenitor cells, or proteins to support the formation of a new endothelial layer in both stents and vascular grafts [9–14]. These surfaces were able to enhance the reendothelialization but none of these studies take along the effect of restenosis by inhibiting the smooth muscle cell migration. In the present study, we aimed to develop a possible stent coating that facilitates EC outgrowth, while it inhibits VSMC migration. Furthermore, procoagulant activity and expression of adhesion molecules on endothelial cells should be minimized. In this study, purified matrix and plasma proteins were studied because these simulate the conditions of a healthy artery and prevent inflammatory reactions due to foreign materials. Elastin is the most abundant extracellular matrix protein in the vascular wall and is formed by cross-linking several tropoelastin molecules which are secreted by VSMCs. Elastin is able to regulate the phenotypic switch and inhibit the proliferation and migration of VSMCs *in vitro* [15–17]. Extracellular matrix protein fibronectin and soluble plasma protein fibrinogen were both shown to facilitate EC adhesion as well as EC and VSMC proliferation and migration [18–20]. Contractile VSMCs cultured on fibronectin have been shown to become more synthetic due to this protein coating [21].

In our study, we aim to develop an optimal possible stent coating, consisting of a cocktail of tropoelastin, fibronectin and fibrinogen to facilitate optimal EC outgrowth and to minimize VSMC proliferation, migration and inflammatory gene expression. Results show that fibrinogen and fibronectin matrix support both favourable EC outgrowth and unfavourable VSMC outgrowth. A tropoelastin surface decreased the proliferation and migration of VSMCs, while it induced an inflammatory and procoagulant response, indicated by excessive expression of VCAM-1, ICAM-1 and IL-8 mRNA in ECs, and increased tissue factor (TF) activity. Our data indicate that a surface coating of fibronectin, fibrinogen and tropoelastin facilitated optimal EC outgrowth, although VSMC outgrowth, inflammatory and procoagulant responses were minimal.

Materials and methods

Protein purification

Human fibronectin was purified from citrated plasma by performing affinity chromatography over a gelatin-Sepharose column as described by Klebe *et al.* [22]. Fibrinogen was purchased from Enzyme Research Laboratories (Swansea, UK). Tropoelastin was made by culturing *Escherichia coli* containing the plasmid for tropoelastin. Cell pellets were lysed with BugBuster (Merck KGaA, Damstadt, Germany). The inclusion bodies were extracted with 6 M urea, 50 mM Tris and 150 mM NaCl pH 7.9. The supernatant was incubated with nickel immobilized metal affinity chromatography (NI-IMAC) resin, washed with 20 mM Imidazole in 6 M urea, 50 mM Tris and 150 mM NaCl pH 7.9. Tropoelastin was eluted with 300 mM Imidazole in 6 M urea, 50 mM Tris and 150 mM NaCl pH 7.9. The fraction was dialyzed against HBSS and analysed by SDS-PAGE for purity.

Single proteins were diluted with PBS to a concentration of 100 μg/ml. Protein mixtures with two proteins contained 50 μg/ml of each protein. The protein mixture containing all three proteins contained 50 μg/ml fibronectin, 45 μg/ml fibrinogen and 5 μg/ml tropoelastin. Surfaces were coated with the different proteins *via* adsorption for 60 min. at room temperature.

Cell culturing

Human umbilical vein endothelial cells were isolated from the umbilical vein. Trypsin-EDTA solution (Invitrogen, Breda, the Netherlands) was added to the vein and incubated for 15 min. at 37°C. The trypsin solution containing the endothelial cells, was flushed out of the vein and cells were spun down for 5 min. at 350 g. Pellet was resuspended in Endothelial Growth Medium-2 (EGM-2; Lonza, Walkersville, MD, USA) and cultured until passage 3.

The VSMCs were isolated from the umbilical cord arteries. The arteries were isolated from the umbilical cord and rinsed with HBS (0.5 mM Hepes, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl) and 200 U/ml pen/strep (Invitrogen). The arteries were dissected into small pieces and plated onto uncoated six-wells plates with the lumen facing down. DMEM (Invitrogen) containing 10% FBS, 100 U/ml pen/strep and L-glutamine (Invitrogen) was added to the wells and refreshed three times a week. After approximately 2 weeks, cells were trypsinized and transferred to a T75 flask in F-12K nutrient mixture (Invitrogen) containing 10% FBS, 100 U/ml pen/strep, 10 mM Hepes (Invitrogen), 10 mM TES (Sigma-Aldrich, Zwijndrecht, the Netherlands), 1× ITS (Sigma-Aldrich), 2.5 μg/ml ascorbic acid and 15 mg ECGS (Sigma-Aldrich). Before using the cells in an assay, the cells were placed on DMEM containing 10% FBS overnight. Cells were used until passage 6.

Migration assay

A 96-wells plate was coated with different protein coatings, air dried and cell seeding stoppers (Platypus Technologies, Madison, WI, USA) were added to each well. HUVECs were added in a concentration of 5×10^5 cells/well in EBM medium containing 0.5% FBS (BioWhittaker Europe, Verviers, Belgium). VSMCs were seeded in a concentration of 2.5×10^5 cells/well in DMEM containing only 0.5% FBS. Cells were able to adhere for 4 hrs and cell seeding stoppers were taken out followed by gently washing with PBS. Fresh cell culture medium containing 0.5% serum was added to the wells and cells were able to migrate for 20 hrs. The cell coating conditions were optimized to reduce variation in cell adhesion. The wells were coated with optimized cell numbers to guarantee a confluent monolayer formation. The optimal conditions were obtained by comparing different cell concentrations. An equal amount of cells was used for all wells to make sure we are only looking at cell migration. The assay was performed in medium with 0.5% FCS, limiting the proliferative capacity of the cells. After this time period, cells were washed, fixed with 2% paraformaldehyde for 20 min., fixed with methanol for 5 min. and stained with May–Grünwald Giemsa. Pictures were made by using a Zeiss inverted microscope with 25× magnification. ImageJ software (National Institute of Health, Bethesda, MD, USA) was used and a macro was programmed that quantified the amount of pixels, which illustrate the absence of cells present in the centre of the well. Pixel intensity gives a reliable indication about the number of cells that migrated into the circle for each well. The pre-migration control (stopper stayed in during this time-point) was set at 0% and the results of all other wells were calculated to this number.

VSMC outgrowth assay

Arteries were isolated from the umbilical cord and rinsed with HBS (0.5 mM Hepes, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl) and 200 U/ml pen/strep. The arteries were dissected into small pieces and plated onto six-wells plates coated with the different proteins with the lumen facing down. Dulbecco's modified Eagle's medicum containing 10% FBS, 100 U/ml pen/strep and L-glutamine was added to the wells and refreshed three times a week. After 12 days, the cross-sectional area of the cell covered surface around the pieces of artery was measured by millimetre paper printed on a transparent sheet.

mRNA isolation and QPCR

The HUVECs and VSMCs were cultured on the different protein coatings in six-wells plates for 24 hrs. mRNA was isolated by using TriPure reagent according to the manufacturer's protocol (Roche, Almere, the Netherlands). After isolation, total RNA was treated with DNAse (GE Healthcare, Hoevelaken, the Netherlands). The presence of genomic DNA was tested by QPCR by using housekeeping gene primers. cDNA was synthesized by using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) and 500 ng of total RNA. Amplification was performed by using 10 μl IQ™ SYBR Green supermix (Bio-Rad) and 10 μl cDNA. HUVEC values were corrected for the amount of β-actin, VSMC values for the amount of GAPDH. Primers used for QPCR were as follows: β-actin forward 5′-gatcggcggctccatcctg-3′ reverse 5′-gactcgtcatactcctgcttgc-3′, GAPDH forward 5′-acagtcagccgcatcttc-3′ reverse 5′-gcccaatacgaccaaatcc-3′, VCAM-1 forward 5′-gctgctcagattggagactca-3′ reverse 5′-cgctcagagggctgtctatc-3′, ICAM-1 forward 5′-ttgaaccccacagtcacctat-3′ reverse 5′-cctctggcttcgtcagaatca-3′, IL-8 forward 5′-ctcttggcagccttcctgatt-3′ reverse 5′-actctcaatcactctcagttct-3′, eNOS forward 5′-gagacttccgaatctggaacag-3′ reverse 5′-gctcggtgatctccacgtt-3′.

Immunofluorescent staining VCAM-1

The HUVECs were cultured in a 24-wells plate coated with the different proteins for 48 hrs in EGM-2 medium. Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. VCAM-1 on the outer membranes was stained with mouse-anti-human VCAM-1 (R&D systems, Abingdon, UK) and counterstained with goat-anti-mouse AF488 (Invitrogen). DAPI containing Prolong gold (Invitrogen) was used to embed the cells. VCAM-1 expression was analysed by using a Zeiss Axiovert40 inverted microscope (Zeiss, Sliedrecht, the Netherlands).

IL-8 ELISA

The HUVECs were cultured in a 48-wells plate coated with the different proteins for 72 hrs in EGM-2 medium. The supernatant was taken off and the IL-8 concentration was measured by using ELISA according to the manufacturer's protocol (human CXCL8/IL-8 ELISA, R&D Systems).

TF activity assay

For analysis of tissue factor (TF) procoagulant activity, HUVECs were cultured in a 96-wells plate on the different protein coatings for 6 hrs. Cells were washed with HBS and incubated with 2 μg/ml factor X (FX, purified from fresh-frozen plasma as described previously [23]), 10 U/ml recombinant factor VIIa (rFVIIa; Novo Nordisk, Alphen aan de Rijn, the Netherlands) 10 mM $CaCl_2$ and 5 mM glucose. FXa generation was measured at 405 nm after the addition of FXa substrate (Pentapharm, Basel, Switzerland). An antibody against TF (Generous gift from Dr. M. Kjalke, Hemostasis Biology, Novo Nordisk, Mavlov, Denmark) was added to block the reaction. Procoagulant activity was calculated based on a standard curve derived from serial dilutions of recombinant TF (Innovin, Dade Behring, Liederbach, Germany).

Whole blood perfusion

Blood samples were drawn from the median cubital vein and anti-coagulated with 10% sodium citrate (3.2% w/v). Glass cover slips were coated and blocked in 1% human serum albumin (ImmunoO fraction V, MP Biomedicals, Amsterdam, the Netherlands) at 4°C overnight. The cover slips were placed on a laminar-flow perfusion chamber and the perfusion chamber was placed upside down in a 37°C water bath. Plasma of the corresponding blood donor was perfused through the tubes and perfusion chamber to rinse. Whole blood was perfused for 30 min. at venous shear rates (300/sec.) or 10 min. at arterial shear rates (1600/sec.). Cover slips were fixed in 4% paraformaldehyde for 30 min., methanol for 5 min. and stained with May–Grünwald Giemsa afterwards. Platelet covered area was quantified by using Leitz microscope and Optimas 6.5 software.

Characterization of the coated surfaces

A total of 96-wells plates were coated with the different protein mixtures followed by blocking with 10% fatty acid free BSA (Sigma-Aldrich). Wells were incubated with antibodies against one of the proteins by using anti-fibrinogen (Santa Cruz, Heidelberg, Germany), anti-fibronectin (R&D Systems) or anti-elastin (Millipore, Amsterdam, the Netherlands). HRP labelled goat-anti-mouse (Dako, Heverlee, Belgium) was added followed by TMB substrate (Tebu-bio, Heerhugowaard, the Netherlands) and H_2SO_4 to stop the reaction. The OD was measured at 450 nm.

Statistics

A Mann–Whitney test in SPSS 15.0 was performed to compare the difference between the two coatings with each other. $N = 3$ for all experiments. Values are presented as mean ± S.E.M. A value of $P < 0.05$ was regarded significant.

Results

HUVEC and VSMCs migration

We investigated the effect of fibronectin, fibrinogen and tropoelastin and mixtures of these proteins on the migration of HUVECs and VSMCs. Migration of HUVECs and VSMCs was investigated by

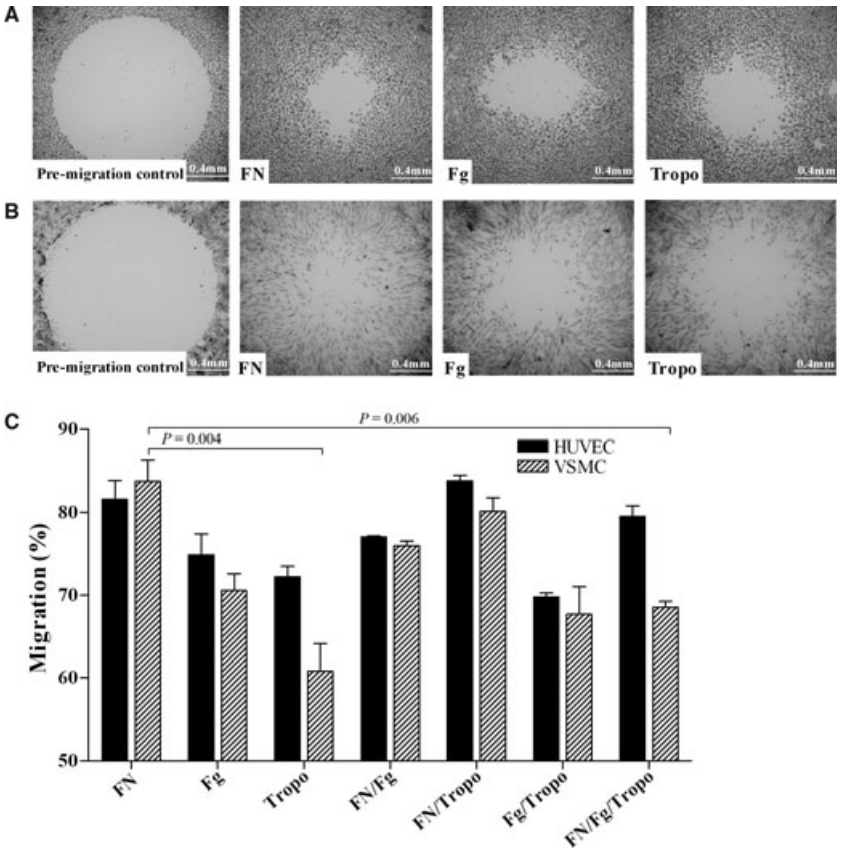

Fig. 1 HUVEC and VSMC migration on different protein coatings. HUVECs (**A**) and VSMCs (**B**) were able to migrate on the different protein coatings towards the empty spot for 20 hrs. A May–Grünwald Giemsa staining was performed to quantify the percentage of migration. The results of the quantification (**C**) show inhibited migration of VSMCs on a tropoelastin (60.8 ± 3.3%) and FN/Fg/Tropo (68.6 ± 0.7%) coating compared to the migration on fibronectin (83.8 ± 2.6%). Experiments were performed with three different primary HUVEC and VSMC donors. Error bars indicate S.E.M.

seeding cells around cell seeding stoppers. When these stoppers were taken out, an empty spot was created to allow cell migration for 20 hrs. A small decrease in HUVEC migration was observed with cells on tropoelastin, with a migration of 72.3 ± 1.3% compared to cells on fibronectin (81.6 ± 2.3%) as shown in Figure 1. A decrease in VSMC migration was clearly observed on a coating with tropoelastin, which resulted in a migration of 60.8 ± 3.3% compared to fibronectin which induced migration for 83.8 ± 2.6%. The mixture of FN/Fg/Tropo resulted in a migration of 68.6 ± 0.7%. These results show that tropoelastin induces less VSMCs migration and that a mixture of FN/Fg/Tropo still contains enough tropoelastin to exert the same effect on the migration.

VSMC outgrowth from umbilical cord arteries

A widely applied method to isolate VSMCs is by placing pieces of artery with the lumen down in a tissue culture dish in DMEM medium containing FBS. After a few days, migrated cells can be observed around the artery which will start to proliferate. With this isolation method, synthetic VSMCs are selected which are highly proliferative and contain less actin compared to the contractile VSMCs. To investigate whether or not the coating can inhibit the migration and proliferation of VSMCs from the artery, the wells were coated with the different protein mixtures and subsequently pieces of artery were added to the wells. After 12 days, the size of each piece of artery and the size of the cell covered area around that piece of artery was measured. The coverage as depicted in Figure 2B is the cell covered area minus the size of the artery in mm^2. Fibronectin was a potent stimulator of the outgrowth resulting in a cell covered area of 31.2 ± 4.0 mm^2 whereas this outgrowth was almost completely abrogated by tropoelastin with an area of 1.6 ± 0.3 mm^2. The mixture of FN/Fg/tropo also had a decreased outgrowth compared to fibronectin with a cell coverage of 8.1 ± 1.5 mm^2. When the phenotype of the cells was examined, elongated and nicely arranged SMCs could be observed from cells on fibronectin and fibrinogen whereas the few SMCs on tropoelastin were disorganized and less vital. These results indicate an inhibited switch from the contractile to the synthetic phenotype by tropoelastin, even in the presence of other proteins.

Inflammatory and adhesion molecule expression levels in HUVECs and VSMCs

Increased expression of adhesion markers such as VCAM-1 and inflammatory markers such as IL-8 enhance migration and adhesion of inflammatory cells from the circulation. To investigate whether or not the expression of these markers is influenced by the surface

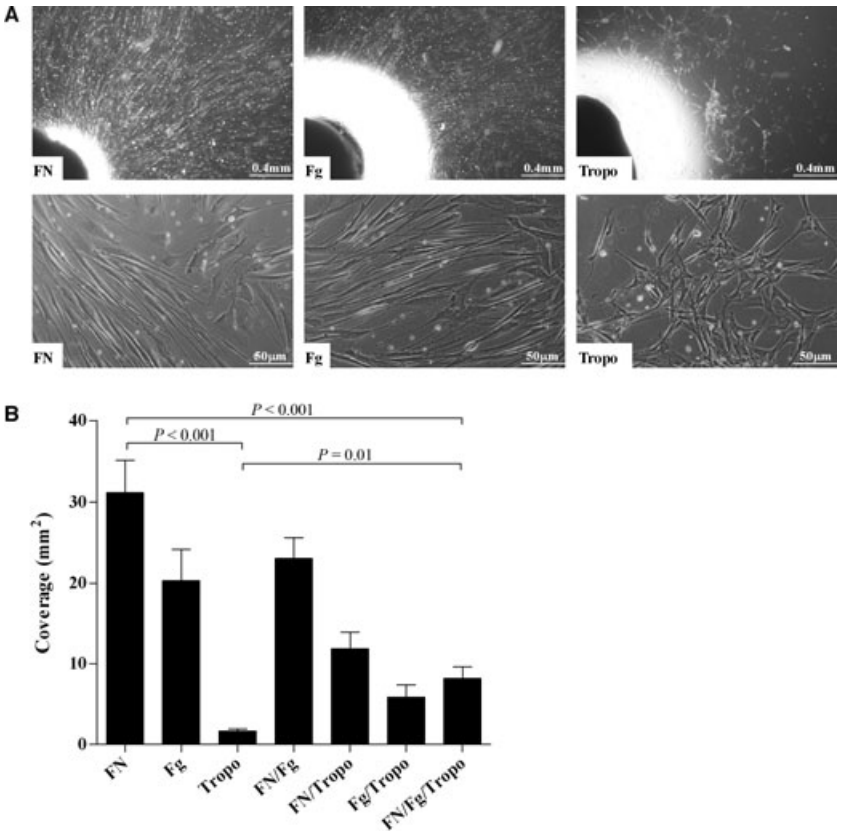

Fig. 2 VSMC outgrowth from the umbilical cord artery on different protein coatings. Pieces of artery were placed lumen down on the different protein coatings and outgrowth of cells from the pieces of artery was measured after 12 days. (**A)** Top row shows outgrowth of cells from the pieces of artery (black in left corner, white circle is microscope light). Bottom row shows higher magnification of the cells with elongated VSMCs on FN and Fg and disorganized VSMCs on tropo suggesting contractile VSMCs. (**B)** Cell covered area was measured in three separate experiments with umbilical cords from three different donors and graphed. Inhibited outgrowth was observed on tropoelastin (1.6 ± 0.3 mm^2) and FN/Fg/Tropo (8.1 ± 1.5 mm^2) compared to fibronectin (31.2 ± 4.0 mm^2). Error bars indicate S.E.M.

coating, mRNA was isolated from HUVECs cultured on the different proteins for 24 hrs and gene expression levels were measured by QPCR. This was the time-point with the most prominent differences observed in a pilot experiment. All values were normalized for the housekeeping gene β-actin. The culturing of HUVECs on tropoelastin resulted in an increased mRNA expression of VCAM-1 of 13.1 ± 4.4 pg/ml compared to fibronectin (0.7 ± 0.2 pg/ml) as shown in Figure 3A. ICAM-1 levels also increased on tropoelastin cultured cells (5.1 ± 1.3 pg/ml compared to FN 0.7 ± 0.2 pg/ml) as well as IL-8 (11.6 ± 3.1 pg/ml compared to FN 2.3 ± 0.5 pg/ml). The increase of these gene expression levels were significantly less on the mixture of FN/Fg/tropo (VCAM-1 3.8 ± 1.9 pg/ml, ICAM-1 1.1 ± 0.2 pg/ml, IL-8 4.3 ± 1.8 pg/ml) but an increase compared to fibronectin was found in all mixtures in which tropoelastin was present. Downregulation of eNOS mRNA levels indicates a dysfunctional endothelium as less NO can be produced by the endothelial cells. eNOS mRNA levels were measured and a trend towards a decrease in eNOS was found when cells were cultured on tropoelastin (2.6 ± 0.4 pg/ml) compared to fibronectin (3.6 ± 0.8 pg/ml). This decrease is gone when cells are cultured on the mixture of FN/Fg/tropo (3.9 ± 0.8 pg/ml) (Fig. S2). All experiments were performed with HUVECs isolated from three different donors. In each experiment, the same variation between the different coatings was observed but the exact values changed between donors due to donor variability, explaining the relatively large error bars. An immunofluorescent staining for membrane VCAM-1 was performed on HUVECs cultured on the different proteins for 48 hrs to examine whether or not the mRNA is translated into functional protein. Fluorescent staining indicates an increased staining intensity for HUVECs on tropoelastin compared to fibronectin as shown in Figure 3B. Also, an ELISA for IL-8 was performed on the supernatant of cells that were cultured on the different coatings for 72 hrs, as shown in Figure 3C. The supernatant of cells cultured on tropoelastin contained 393.3 ± 63.8 pg/ml IL-8 which is a high increase compared to the IL-8 levels measured in the supernatant of cells cultured on fibronectin (95.9 ± 11.1 pg/ml). Culturing HUVECs on a mixture of FN/Fg/Tropo did not give an increase in IL-8 (124.3 ± 19.5 pg/ml). These results are comparable to the differences found in IL-8 mRNA expression levels and indicate effective translation of the mRNA into protein. Besides HUVECs, also VSMCs were cultured on the different proteins for 24 hrs and mRNA was isolated. All VSMC values were normalized for the housekeeping gene GAPDH, as β-actin levels might be affected by the phenotype switch. QPCR indicated no significant differences in VCAM-1, ICAM-1 and IL-8 expression in these cells (Fig. S3). These results show that culturing HUVECs on tropoelastin leads to an increased inflammatory and adhesion marker expression, although VSMC gene expression levels are not influenced by the tropoelastin coating.

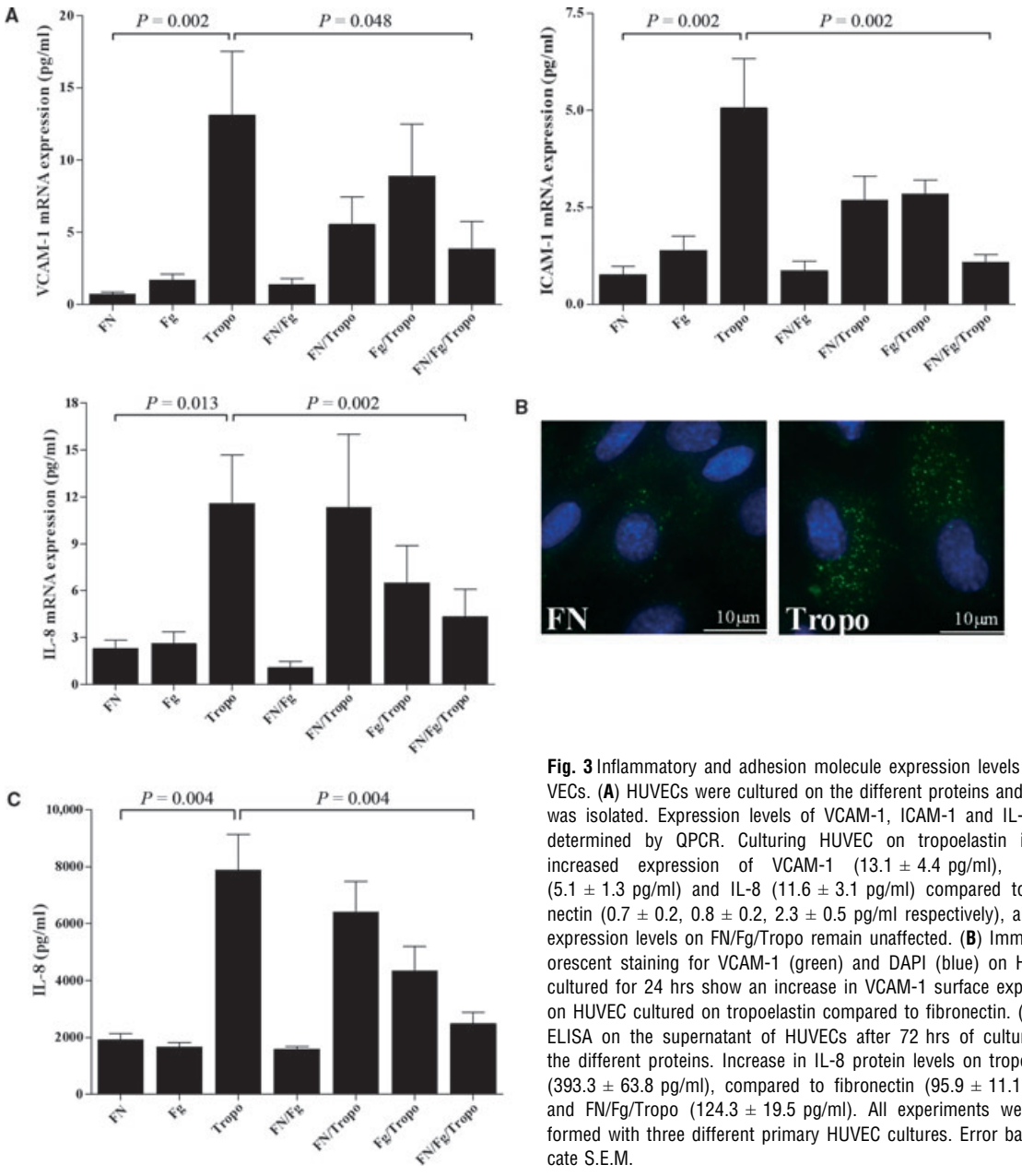

Fig. 3 Inflammatory and adhesion molecule expression levels in HUVECs. (**A**) HUVECs were cultured on the different proteins and mRNA was isolated. Expression levels of VCAM-1, ICAM-1 and IL-8 were determined by QPCR. Culturing HUVEC on tropoelastin induces increased expression of VCAM-1 (13.1 ± 4.4 pg/ml), ICAM-1 (5.1 ± 1.3 pg/ml) and IL-8 (11.6 ± 3.1 pg/ml) compared to fibronectin (0.7 ± 0.2, 0.8 ± 0.2, 2.3 ± 0.5 pg/ml respectively), although expression levels on FN/Fg/Tropo remain unaffected. (**B**) Immunofluorescent staining for VCAM-1 (green) and DAPI (blue) on HUVECs cultured for 24 hrs show an increase in VCAM-1 surface expression on HUVEC cultured on tropoelastin compared to fibronectin. (**C**) IL-8 ELISA on the supernatant of HUVECs after 72 hrs of culturing on the different proteins. Increase in IL-8 protein levels on tropoelastin (393.3 ± 63.8 pg/ml), compared to fibronectin (95.9 ± 11.1 pg/ml) and FN/Fg/Tropo (124.3 ± 19.5 pg/ml). All experiments were performed with three different primary HUVEC cultures. Error bars indicate S.E.M.

Procoagulant activity on HUVECs

In addition to increased inflammation, a procoagulant state of HUVECs is also an unfavourable characteristic of cells on a stent surface. Under normal physiological conditions, endothelial cells have an anticoagulant surface and do not express TF. Under inflammatory conditions, TF can be exposed leading to complex formation with factor VIIa resulting in activation of factor X and thereby the initiation of the extrinsic pathway of coagulation. To examine whether or not the coating induces expression of active TF on the surface of HUVEC, factor Xa formation was measured after the addition of factor X, recombinant factor VIIa and calcium. The amount of Xa is dependent on the concentration active TF present on the cell surface. Figure 4 shows that an increased TF activity was measured on HUVECs cultured on tropoelastin (511.6 ± 26.7%) compared to fibronectin (100 ± 3.9%) whereas no differences were found on the mixture of FN/Fg/tropo (76.3 ± 25.0%). An antibody against TF blocked the Xa formation completely

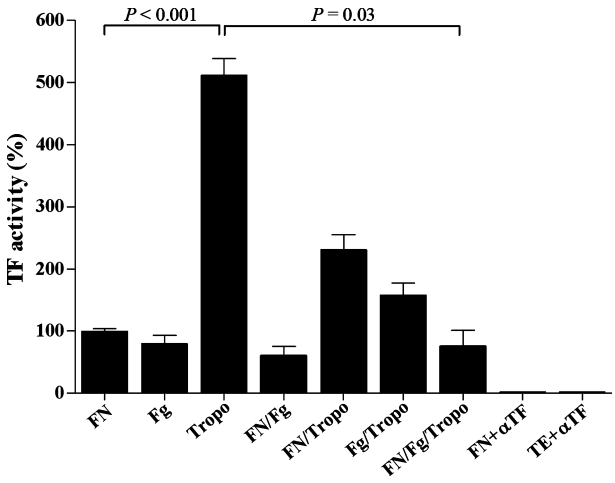

Fig. 4 TF activity on HUVECs. TF activity on the outer membrane of HUVECs was measured after 6 hrs of culturing on the different proteins. Increased TF activity was measured on cells cultured on tropoelastin (511.6 ± 26.7%) compared to cells cultured on fibronectin (100 ± 3.9%) or FN/Fg/Tropo (76.3 ± 25.0%). An antibody against TF (αTF) added to the cells blocked the reaction. Experiments were performed with three different primary HUVEC cultures. Error bars indicate S.E.M.

indicating that it was TF from the cells resulting in factor Xa formation. These results show an increased procoagulant state of HUVECs when cultured on tropoelastin.

Platelet adhesion under flow conditions

Platelets are known to adhere to fibronectin and fibrinogen under flow conditions. To analyse the amount of adhered platelets to the different coatings, citrate anti-coagulated blood was perfused over coated cover slips in a laminar-flow perfusion chamber. Blood was perfused at either venous shear rates (300/sec., Fig. 5A) for 30 min. or arterial shear rates (1600/sec., Fig. 5B) for 10 min and adhered cells were fixed and stained with May–Grünwald Giemsa. The platelet covered area was quantified and the results show that platelet adhesion is abrogated in the presence of tropoelastin (0%) under both shear rates. Fibrinogen is the most adhesive substrate and shows high surface coverage under both venous and arterial shear rates, 72 ± 8% and 84 ± 6%, respectively. Platelets perfused over fibronectin adhere well under lower shear rates, 51 ± 3%, but the coverage is decreased with high shear rates to 13 ± 3%. Also the mixture with FN/Fg/tropo supports platelet adhesion better under venous shear rates, 75 ± 13%, compared to arterial shear rates, 37 ± 8%. These results show decreased platelet adhesion in the presence of tropoelastin but still a reasonable amount of adhesion on the mixture of FN/Fg/tropo.

Discussion

In this study, we have characterized the effect of fibrinogen, fibronectin and tropoelastin coatings on endothelial cell and smooth muscle cell growth and function. Tropoelastin has both favourable

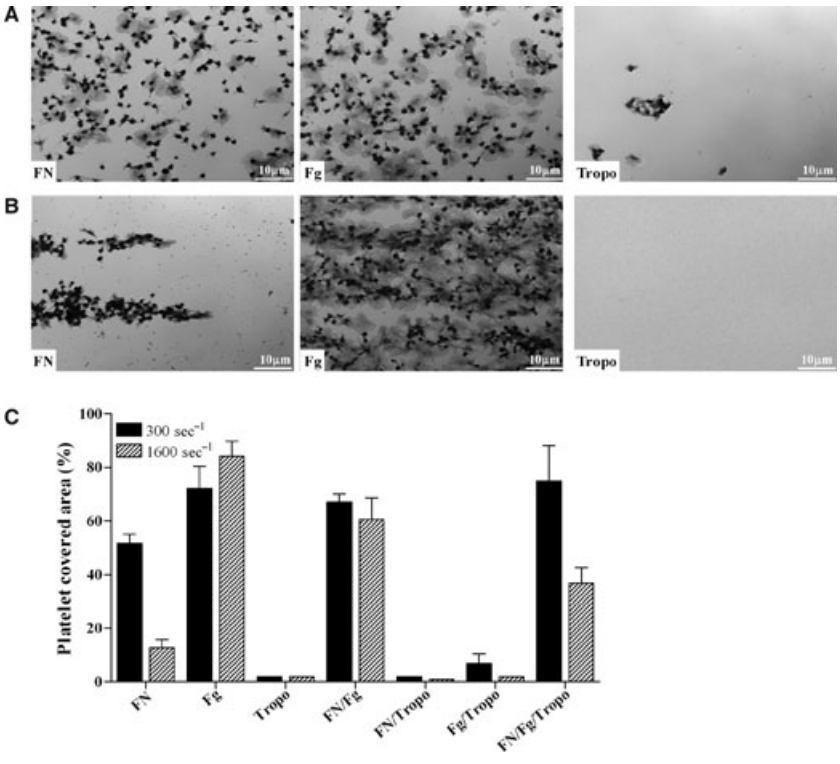

Fig. 5 Platelet covered area on different protein coatings. (**A**) Adhered platelets were stained with May–Grünwald Giemsa after 30 min. of perfusion of citrated whole blood under venous shear rates (300/sec.) and (**B**) after 10 min. of perfusion under arterial shear rates (1600/sec.). (**C**) Quantification of platelet covered area of three different blood donors. Platelets were not able to adhere to tropoelastin (0%) but adhered to the mixture of FN/Fg/Tropo under both venous (75 ± 13%) and arterial (37 ± 8%) shear rates. Experiments were performed with three different blood donors. Error bars indicate S.E.M.

Table 1 Summary results of different protein coating properties.

	FN	Fg	Tropo	FN/Fg	FN/Tropo	Fg/Tropo	FN/Fg/Tropo
HUVEC migration	+	+	+	+	+	+	+
VSMC migration	+	–	–	+	+	–	+/–
VSMC outgrowth	+	+	–	+	+/–	–	–
VCAM-1, ICAM-1, IL-8 expression HUVEC	–	–	+	–	+/–	+/–	–
VCAM-1, ICAM-1, IL-8 expression VSMC	–	–	–	–	–	–	–
VCAM-1, IL-8 protein levels	–	–	+	–	+	+/–	–
TF activity	–	–	+	–	+/–	+/–	–
Platelet adhesion venous shear	+	+	–	+	–	–	+
Platelet adhesion arterial shear	+/–	+	–	+	–	–	+/–

The effects of the different coatings in the *in vitro* assays are summarized in the table. When the coating shows an increased effect in the corresponding assay, it is indicated with a "+". When the coating shows a decrease in the specific assay, it is indicated with a "–".

and unfavourable properties. On one hand, a tropoelastin matrix decreases the smooth muscle cell migration, while on the other hand, it does not support an effective EC outgrowth. The ECs that adhered to tropoelastin were proinflammatory and procoagulant, which indicates that tropoelastin matrix by itself is not an optimal stent coating. Fibrinogen and fibronectin matrix support EC outgrowth, but do also allow unfavourable VSMC outgrowth. Our data, summarized in Table 1, indicate that a mixture of FN/Fg/tropo supported EC migration without triggering inflammation and coagulation, although VSMC migration was minimized.

Stent coatings capturing circulating endothelial progenitor cells (EPC) or promoting endothelial cells growth have been shown to be successful with regard to improved reendothelialization. Antibodies against CD34 or VE-cadherin coated on the stent struts increased endothelial progenitor cell adhesion and thereby the formation of a new endothelial layer [9–11, 14, 24]. RGD-peptides and a REDV-epitope recognizing endothelial cell specific integrins, were shown to activate endothelial cell spreading and proliferation improving reendothelialization [12, 25] However, restenosis is not always prevented by the improved formation of an endothelial lining and none of these studies take along the effect of restenosis by inhibiting the smooth muscle cell migration. Smooth muscle cells are still activated by the stent placement and will still be able to contribute to neointimal hyperplasia. Drug-eluting stents are able to prevent this, but delay the healing of the artery and thereby increase the risk for in-stent thrombosis.

In this study, we not only characterized the possible stent coatings on endothelial cell growth but also on their capability to inhibit smooth muscle cell migration. *In vivo,* the coating should provide an optimal surface for endothelial cells although smooth muscle cell migration should be inhibited when the cells come into close proximity of the stent struts. Elastin and tropoelastin are known for their capacity to inhibit VSMC proliferation and migration [15–17]. Our results show that tropoelastin has a similar effect as the main molecule elastin and is also suitable for the inhibition of VSMC migration. Tropoelastin can also promote endothelial cell proliferation when coated onto a stainless steel surface compared to an uncoated surface [26]. Conflicting results showed that endothelial cells were unable to form a monolayer on tropoelastin coated surfaces due to the decreased ability to adhere to the protein [27]. Our results support the results of Yin *et al.* [26] and show that tropoelastin is indeed able to induce endothelial cells proliferation but to a lesser extent as fibronectin and fibrinogen. Also, cell adhesion to tropoelastin in our experiments was not decreased as observed by Williamson *et al.* [27]. Besides the limited capacity of tropoelastin for EC outgrowth, we also observed that tropoelastin induced increased inflammatory gene expression and increased procoagulant activity indicates an unfavourable complication of tropoelastin matrix. A fibrinogen coating was shown by previous studies to enhance endothelial cell adhesion and SMC proliferation and migration [18, 20] and also this was supported by our data. Fibronectin is known as a potent stimulator of cell adhesion and growth. Previous studies showed its ability to stimulate the differentiation of contractile VSMCs into a synthetic state and to promote proliferation and migration [19, 21].

Another characteristic of fibronectin and fibrinogen is that both these proteins are capable of capturing platelets under flow conditions [28, 29]. In our hands, fibrinogen was the strongest platelet adhesive surface under both arterial and venous shear rates. No platelet adhesion and spreading were observed on tropoelastin and when tropoelastin was mixed with fibrinogen or fibronectin, adhesion was almost completely inhibited. The mixture of FN/Fg/tropo was still able to obtain an almost 40% platelet covered surface under arterial shear rates. Many studies focus on the suppression of platelet adhesion to the stent surface as this would lead to thrombosis and finally to occlusion of the stent. Recent studies, however, have shown that platelets might be necessary for reendothelialization. Platelets are a rich source of cytokines and growth factors that can induce differenti-

ation of EPCs towards mature endothelial cells, and platelets have the capacity to facilitate homing of ECs and EPCs towards injury [30–36]. Moreover, activated platelets express P-selectin which binds P-selectin glycoprotein ligand-1 (PSGL-1) on EPCs resulting in tethering and rolling of these cells on the activated platelets [33, 36]. A coating of platelet adhesive proteins on a stent surface that captures only a monolayer of platelets might facilitate the reendothelialization process by providing a source of growth factors and an extra capturing mechanism.

It is unknown how the coating would be modified by plasma proteins upon stent implantation. The platelet adhesion experiments were performed with whole blood so the proteins present in the blood were able to bind to the surface and influence our own protein coatings. Plasma fibrinogen is known to bind immediately to the surface [37]. However, a diminished platelet adhesion in the presence of tropoelastin could still be observed indicating that our coating is still present on the surface. A major limitation of this study is the use of plastic- and glass-coated surfaces. Proteins binding to the metal surface of a stent are known to exhibit a different structure [38]. *In vivo* stent placement in a large animal model should further indicate the efficacy of this stent coating.

Taken together, a stent coating with the natural matrix and plasma components fibronectin, fibrinogen and tropoelastin, could be a favourable coating that facilitates endothelial cells growth and one that inhibits the negative side effects of smooth muscle cells activation. This stent may be an alternative for drug-eluting stents, which also inhibit the activation of VSMCs but do not allow reendothelialization, because EC proliferation is blocked. *In vivo* experiments are required to investigate whether or not this coating indeed leads to reendothelization and reduced restenosis. This stent coating might represent a novel therapeutic approach for improving the efficiency and long-term safety of stent placement in cardiovascular disease patients.

Acknowledgements

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project CIRCULATING CELLS [grant 01C-102], and supported by the Netherlands Heart Foundation.

The research was performed by C.T. and E.M-N. C.T. analysed the data and wrote the article. M.R., E.L., I.H., P.G. and G.P. were responsible for participating in the discussion of all results and revising the paper critically.

Conflict of interest

Dr. E. Ligtenberg is an employee at OrbusNeich.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Characterization of the coated surfaces. Tissue culture treated 96-wells plates were coated with the different proteins. By antibodies against the different proteins, the presence of all the proteins was established (fibronectin top, fibrinogen middle, tropoelastin bottom graph). The highest OD450 was measured in wells were only one protein in a concentration of 100 μg/ml was coated. When a mixture of two proteins was coated, a decreased OD450 was measured and the results indicate the presence of both proteins in an equal manner. The mixture of three proteins also shows the presence of all three proteins after coating. As only 5 μg/ml tropoelastin was used in this mixture, a lower OD450 is measured for this protein. Experiment was performed three times. Error bars indicate S.E.M.

Figure S2. eNOS mRNA expression in HUVECs. HUVECs were cultured on the different proteins and mRNA was isolated. Expression levels of eNOS were determined by QPCR. Culturing HUVEC on tropoelastin reduced the expression of eNOS (2.6 ± 0.4 pg/ml) compared to fibronectin (3.6 ± 0.8 pg/ml), while expression levels on FN/Fg/Tropo remain unaffected (3.9 ± 0.8 pg/ml). Experiment was performed with three different primary HUVEC cultures. Error bars indicate S.E.M.

Figure S3. Inflammatory and adhesion molecule expression in HA-VSMCs. HA-VSMCs were cultured on the different proteins and mRNA was isolated. Expression levels of VCAM-1, ICAM-1 and IL-8 were determined by QPCR. No significant differences were observed between the coatings on gene expression levels. All experiments were performed with three different primary HUVEC cultures. Error bars indicate S.E.M.

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