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Porcine Endothelial Cells Cocultured with Smooth Muscle Cells Became Procoagulant *In Vitro*

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Endothelial cell (EC) seeding represents a promising approach to provide a nonthrombogenic surface on vascular grafts. In this study, we used a porcine EC/smooth muscle cell (SMC) coculture model that was previously developed to examine the efficacy of EC seeding. Expression of tissue factor (TF), a primary initiator in the coagulation cascade, and TF activity were used as indicators of thrombogenicity. Using immunostaining, primary cultures of porcine EC showed a low level of TF expression, but a highly heterogeneous distribution pattern with 14% of ECs expressing TF. Quiescent primary cultures of porcine SMCs displayed a high level of TF expression and a uniform pattern of staining. When we used a two-stage amidolytic assay, TF activity of ECs cultured alone was very low, whereas that of SMCs was high. ECs cocultured with SMCs initially showed low TF activity, but TF activity of cocultures increased significantly 7–8 days after EC seeding. The increased TF activity was not due to the activation of nuclear factor kappa-B on ECs and SMCs, as immunostaining for p65 indicated that nuclear factor kappa-B was localized in the cytoplasm in an inactive form in both ECs and SMCs. Rather, increased TF activity appeared to be due to the elevated reactive oxygen species levels and contraction of the coculture, thereby compromising the integrity of EC monolayer and exposing TF on SMCs. The incubation of cocultures with N-acetyl-cysteine (2 mM), an antioxidant, inhibited contraction, suggesting involvement of reactive oxygen species in regulating the contraction. The results obtained from this study provide useful information for understanding thrombosis in tissue-engineered vascular grafts.

Introduction

Thrombosis is a limiting factor for the development of small-diameter vascular grafts. Endothelial cells (ECs) that line the innermost part of the blood vessel provide an antithrombogenic surface that prevents thrombosis by constitutively expressing thrombomodulin (TM), prostacyclin (PGI_2), and nitric oxide.[1] Therefore, EC seeding on vascular grafts represents a promising approach to improve patency. However, until now, EC seeding has had only limited success in expanded polytetrafluoroethylene vascular grafts with a diameter greater than 6 mm.[2] In small-diameter (<4 mm) vascular grafts, there is a high rate of thrombosis. During the inflammatory response, ECs shift the hemostatic balance in favor of thrombosis by expression of procoagulant molecules such as tissue factor (TF),[3] von Willebrand factor, and plasminogen activator inhibitor.[4] There is some evidence suggesting that the seeded ECs display normal function by the presence of TM and reduced thrombogeneity.[5,6] However, other lines of evidence suggest the opposite; human ECs seeded on expanded polytetrafluoroethylene vascular graft have been reported to release growth factors, platelet-derived growth factor, and basic fibroblast growth factor, which cause smooth muscle cell (SMC) proliferation, neointimal fibrous hyperplasia, and graft failure.[7] Human umbilical vein ECs (HUVECs) seeded on Dacron (polyethylene terephthalate) showed less-effective adherence[8] and increased E-selectin expression that favors leukocyte adhesion.[9] HUVEC seeding on albumin- and chitosan-coated polyester fabric released von Willebrand factor and expressed low TM surface activity, despite maintaining an adequate response to human thrombin and dibutyryl-cAMP.[10]

TF is the key initiator of the coagulation cascade *in vivo*. The binding of TF to factor VII forms a TF/VIIa complex that subsequently activates factor X, which ultimately converts prothrombin to thrombin, resulting in blood coagulation.[11] TF is normally absent on the luminal surface of vascular ECs *in vivo*, but TF gene expression in ECs and SMCs is stimulated by inflammatory agents such as lipopolysaccharide (LPS) and tissue necrosis factor alpha.[12]

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The mechanisms regulating EC phenotype (procoagulant or anticoagulant) have not been fully defined. Inflammation is associated with thrombosis, and nuclear factor kappa-B (NFκB) is generally regarded as a key initiator of inflammation. It is therefore possible that the activation of NFκB might be responsible for the increased TF activity shown in ECs. Recent studies suggest that increased levels of reactive oxygen species (ROS), such as superoxide, H_2O_2, and peroxynitrite, are important contributors to inflammation and many cardiovascular diseases.[13,14]

In a previous study,[15] we established a direct coculture model wherein a confluent porcine EC monolayer was cultured on a quiescent porcine SMC layer, which simulated the physical proximity of ECs and SMCs that occurs in small arteries and arterioles *in vivo*. Direct coculture also served as a simplified model to study EC seeding on engineered vascular grafts that contain SMCs. Although SMCs do not produce a true basement membrane for such short-term culture, the ECs adhere to a layer of extracellular matrix consisting of fibronectin, collagen I, and collagen III.[16] Preliminary results demonstrated that this coculture resulted in altered porcine EC function, including increased acetylated low density lipoprotein (Ac-LDL) uptake, and decreased platelet EC adhesion molecule (PECAM) expression. Further, the low serum conditions used inhibited proliferation of porcine[15] and human cells.[17] In the current study, we tested the hypotheses that (1) failure of porcine EC seeding in tissue-engineered vascular grafts (TEVGs) to prevent thrombosis is due to a phenotypic change of the graft cells that leads cells to increased TF activity and (2) increased ROS levels and activated NFκB are responsible for the procoagulant phenotype. We examined TF expression and TF activity on ECs, SMCs, and EC/SMC cocultures. We also examined the roles of NFκB and ROS in TF activation.

Materials and Methods

Antibodies

Murine monoclonal anti-human TF (mAB #4509) was purchased from American Diagnostica (Greenwich, CT). Mouse monoclonal anti-porcine CD31 (PECAM) was obtained from Antigenix-America (Huntington Station, NY). Mouse monoclonal NFκB p65 (sc-8008) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Porcine SMCs were isolated from aortic explants of Yucatan miniature swine or farm pigs, as previously described.[15] All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee. ECs were scraped directly from the arterial intima. SMCs were cultured in the proliferative medium (PM) that consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Gibco), 5% porcine serum (PS; Gibco), 0.05 g/L vitamin C, 3×10^{-6} g/L $CuSO_4$, 0.05 M HEPES, 0.05 g/L proline, 0.05 g/L alanine, 0.02 g/L glycine, 10×10^{-9} g/L basic fibroblast growth factor, 10×10^{-9} g/L platelet-derived growth factor, and 1×antibiotic/antimycotic (all from Sigma, St. Louis, MO). ECs were cultured with low-glucose DMEM supplemented with 5% FBS, 5% PS, 1.25 μg/mL heparin, and 1×penicillin G. Cells of passage 3–6 were used in all experiments. All cells were incubated at 37°C and 5% CO_2.

EC/SMC coculture model

The EC/SMC direct coculture model was developed as described previously.[15] Briefly, a basal adhesion protein (fibronectin at 5 μg/cm^2 or laminin at 2 μg/cm^2) was adsorbed onto six-well plates (area = 9.6 cm^2; Becton Dickson, Franklin Lakes, NJ), and then SMCs were cultured in PM to confluence. Quiescent medium (QM) consisting of DMEM/F12 (Gibco) supplemented with 1×insulin, transferrin, and selenium supplement (Gibco), and 0.05 g/L vitamin C was used to induce a quiescent state in SMCs. On the basis of our previous study, at least 2 days were needed to shift SMCs to a quiescent state.[15] Fibronectin was then added above SMCs for 4 h at 37°C. ECs were then seeded at 60,000–100,000 cells/cm^2 onto SMCs. Although SMCs can survive in serum-free media, ECs require low levels of serum. Thus, the cells were cultured in a coculture medium that consisted of one part PM and two parts QM to yield a final serum content of 3.3% (1.67% FBS and 1.67% PS). This culture condition maintained the quiescent state of SMCs and normal morphology of ECs.[15] All experiments were performed at least 2 days after the coculture was initiated. To determine the effect of antioxidants on the coculture system, cells were incubated with 1, 2, or 5 mM N-acetyl-L-cysteine (NAC; Sigma) during the culture period.

TF procoagulant activity assay

The surface TF activity was measured using a two-stage amidolytic assay (Roche Applied Science, Indianapolis, IN).[18] Cultures were rinsed with phosphate-buffered saline (PBS) twice, and 0.5 mL of reaction buffer (0.5 μg/mL Factor VIIa, 10 μg/mL Factor X, 50 mM Tris, 150 mM NaCl, 2 μg/mL aprotinin, and pH 8.3) was added and incubated for 15 min. TF on the cell surface bound to factor VIIa to form a TF/VIIa complex that activated factor X to factor Xa. In the next stage, 0.5 mL reaction buffer was transferred to a disposable cuvette and 50 μL substrate Chromozym X (2 mM) was added. The chromogen was cleaved by factor Xa to produce chromogenic p-NA that was immediately measured at 405 nm. The ΔA_{405}/min was measured using SmartSpectra 3000 (Bio-Rad, Hercules, CA). Lipidated TF (American Diagnostica) and Factor Xa (Haematologic Technologies, Essex Junction, VT) were used to generate standard curves. Standard curves were calibrated for each set of assay.

Immunostaining

TF immunostaining. Cells were rinsed twice with Dulbecco's PBS, and fixed using 4% paraformaldehyde for 10 min. After rinsing cells twice with PBS, the cells were incubated with 10% goat serum in PBS for 30 min at 37°C. Primary murine anti-human TF antibody (dilution 1:100 in blocking buffer) was added and incubated at 4°C overnight. After rinsing with PBS, secondary antibody linked to Alexa 546 (1:200; Invitrogen, Carlsbad, CA) was added and incubated for another hour. Then, the cells were postfixed for 15 min using paraformaldehyde solution, rinsed with PBS, mounted, and viewed using fluorescent microscopy. For TF staining, the permeabilization step was omitted to measure

cell surface TF. To observe ECs, after the secondary fixation with paraformaldehyde, the cell monolayer was incubated with mouse anti-porcine PECAM antibody that was directly labeled with Alexa Fluor 488 using the Zenon mouse IgG1 labeling kit (Invitrogen), according to the manufacturer's protocol.

For long-term tracking of living cells, CellTracker Green (CTG; Invitrogen) was used to label SMCs. SMCs were incubated with 10 μM CTG for 45 min at 37°C. The medium was exchanged with the CTG-free medium, and cells were allowed to incubate for another 30 min before seeding EC to facilitate the modification of CTG by intracellular thiols and esterases, making the molecule cell-impermanent and fluorescent. CTG was detected with a standard fluorescein isothiocyanate filter set using fluorescent microscopy on a confocal microscope.

NFκB p65 immunostaining. Staining for the NFκB subunit p65 was used to assess nuclear localization of NFκB that represents the activated form of NFκB. This mouse anti-human monoclonal antibody was labeled directly with the Alexa Fluor 488 Zenon IgG1 labeling kit (Invitrogen). The fraction of nuclei positive for p65 was then used as a measure of the cells in which NFκB was activated.

Viability/cytotoxicity. Cell viability was determined using a LIVE/DEAD Viability/Cytotoxicity kit from Invitrogen. Live cells incorporate calcein AM (494/517 nm) and produce green fluorescence. Ethidium homodimer-1 (EthD-1, 528/617 nm) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, producing a bright red fluorescence. Two-channel scanning was conducted using confocal microscopy. As a positive control, cells were incubated with 100 μM LPS for 4 h before the assay.

Confocal microscopy and image processing

A Confocal Laser Scanning Microscope (LSM 510; Carl Zeiss, Thornwood, NY) with a 40×/0.8 water-immersion objective was used to capture images. CTG and Alexa Fluor 488 were excited with a 488 nm laser; EthD-1 and Alexa Fluor 546 were excited with a 541 nm laser. Z-sections of 1 μm thickness were obtained to detect EC and SMC layers in coculture labeled with PECAM and CTG, respectively. Matlab Image Processing (MathWork, Natick, MA) was used to calculate the image intensity by averaging the pixel grayscale values. To compare the intensity, the scanning configurations were fixed (laser intensity, laser output, pinhole side, gain, offset, amplifier, scanning speed, and filter set) for all experiments with a specific antibody or fluor. The multitrack mode was chosen in conjunction with the filter sets to exclude overlap between fluorescent signals.

Statistical analysis

All the data are expressed as mean ± standard deviation. Student's *t*-test was used to compare the difference between two groups. *p*-Values less than 0.05 were considered significant.

Results

TF expression on ECs, SMCs, and in coculture

Immunostaining of TF shows that TF expression was uniform on quiescent SMCs (Fig. 1A), and heterogeneous on confluent ECs (Fig. 1B). To locate the position of TF staining on ECs, the EC monolayer was further stained with PECAM (Fig. 1C). Since PECAM is localized at the borders of confluent ECs, PECAM staining identifies individual cells. The staining with both TF and PECAM indicated that 14 ± 5% of ECs expressed TF at a level similar to that of SMCs, whereas the remaining ECs expressed levels of TF that were not different from background. The average intensity of SMC TF staining on captured images was 133 ± 19 ($n = 10$ images and a grayscale from 0 to 255), whereas the average intensity on ECs was 50 ± 6 ($n = 10$ images).

To determine TF expression of ECs and SMCs in coculture, SMCs were prestained with CTG, and then cocultures were immunostained for TF (Alexa 546). Z-sectioning of the coculture yielded intensity profiles for CTG and Alexa 546 (TF on ECs and SMCs). Figure 2 shows the intensity profile. The peak of the green intensity corresponded to the position of SMCs, which peaked 8 μm from the bottom of stack. On the basis of our previous study,[15] the center of the EC monolayer should be at 13 μm away from the center of SMCs. These

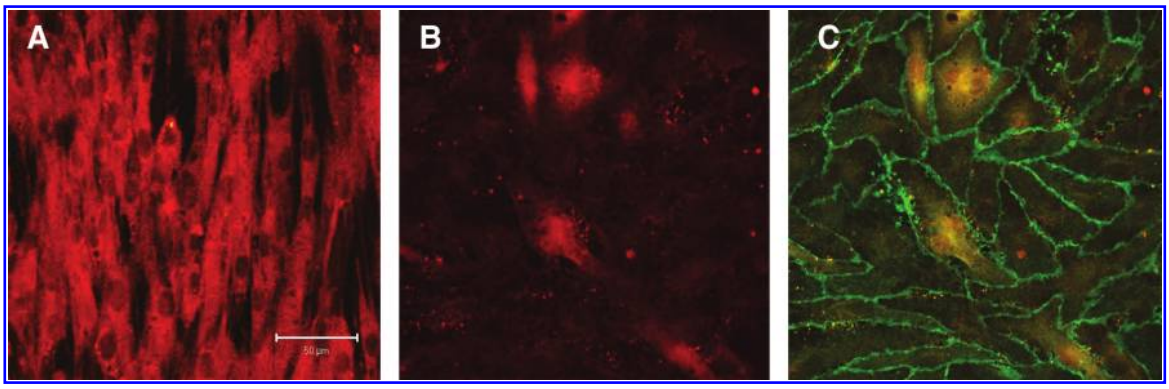

FIG. 1. TF expression on cultured quiescent SMC in the quiescent medium (**A**) and on confluent EC in 3.3% serum (1.67% fetal bovine serum and 1.67% porcine serum) (**B**). (**C**) shows TF in red overlaid with platelet EC adhesion molecule in green. Scar bars: 50 μm. Cell density for ECs is 64,700 ± 12,600 cells/cm^2. TF, tissue factor; SMCs, smooth muscle cells; EC, endothelial cell. Color images available online at www.liebertonline.com/ten.

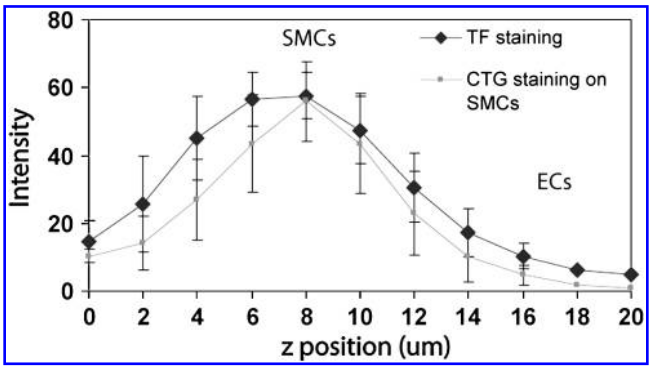

FIG. 2. Localization of TF expression on EC/SMC coculture system. SMCs were prestained with CTG before the seeding of ECs. ECs and SMCs were cocultured in 3.3% serum for 2 days, and then incubated with TF monoclonal antibody, followed by a secondary antibody labeled with Alexa 546. Z-section was conducted using Zeiss Confocal Microscopy LSM 510. The intensity at each z position was calculated by Matlab Image processing toolbox. CTG, CellTracker Green.

results indicate that most of the TF staining is associated with SMCs.

TF activity on ECs, SMCs, and EC/SMC coculture systems

Since TF antigen expression on the cell surface does not necessarily correlate with TF functional activity,[19] we measured TF activity on confluent ECs, quiescent SMCs, and EC/SMC cocultures. Serum in the culture medium had a significant effect on measured TF activity. Table 1 lists the TF activity on SMCs, ECs, and coculture. On average, TF activity on SMCs cultured in the coculture medium with 3.3% serum, as described in Materials and Methods section, was higher than that in QM with no serum (0.6 vs. 0.27), but due to the high variability there was no statistical difference between these two groups ($p > 0.1$, Table 1). Since ECs cultured alone cannot survive in the serum-free medium, we cultured ECs with the coculture medium containing 3.3% serum, and the measured TF activity by ECs was 0.05. This value was at the same level as that of the coculture, suggesting that coculture TF activity was determined by the top EC monolayer (0.05 ± 0.01), and the presence of ECs either inhibited or blocked SMC TF activity (0.6 ± 0.32). In the coculture system, TF activity was higher when no serum was used (0.15 ± 0.04), as compared to when 3.3% serum was used (0.05 ± 0.01, $p < 0.05$). Therefore, the coculture medium that consists of 3.3% serum is better than QM for EC/SMC coculture.

Table 1. Effect of Serum on Tissue Factor Activity (ΔA_{405}/min)

Condition	*SMCs*	*Coculture*	*ECs*
QM no serum	0.27 ± 0.03	0.15 ± 0.04	—
Coculture medium 3.3% serum	0.6 ± 0.32	0.05 ± 0.01	0.05
t-Test between two groups	0.34	0.04	

SMCs were cultured in QM for 2 days, after which one set of SMCs were cultured on QM for an additional 2 days and the other group of SMCs were cultured in the coculture medium for 2 days. TF activity was determined at the end of the incubation. TF activity in ECs cultures was determined 2 days after culture in the coculture medium. No test was performed on ECs cultured in QM as ECs did not survive.

SMCs, smooth muscle cells; ECs, endothelial cells; QM, quiescent medium; TF, tissue factor.

The method of cell monolayer detachment influenced TF activity. Commonly used methods to assay TF activity include assay of intact monolayers,[19–22] or assay of monolayers that are detached either by scraping[23] or by treatment with trypsin/ethylenediaminetetraacetic acid.[24] Ryan and coworkers showed that TF is not expressed on the apical surface of an intact stimulated HUVEC monolayer.[25] Mulder *et al.* reported that 95% of total TF activity was expressed at the basolateral aspects of HUVEC.[22] Table 2 shows the effect of treatments on measured TF activity of EC monolayers. As expected, TF activity on intact monolayers showed the least activity, and all other treatments were normalized to TF activity on the intact monolayer. Scraping the monolayer exposed TF at the basolateral side and increased TF activity by 9.6-fold, whereas trypsin treatment increased EC TF activity by only 3-fold. It is likely that trypsin treatment cleaves some TF molecules on the EC basolateral surface, thereby reducing TF activity. Since TF activity on the intact coculture is the most relevant to the thrombogenicity of TEVGs, it was the primary assessment used in this study.

Using intact cell layer and the coculture medium with 3.3% serum content, we measured the TF activity of ECs, SMCs, and cocultures over the culture period, as illustrated in Figure 3. ECs displayed almost undetectable TF activity during the whole culture period, which is consistent with observations *in vivo*[26] and *in vitro*.[27] SMCs cultured in the low-serum medium alone expressed a high level of TF activity that decreased over time until it dramatically increased after 6 days of culture. In the EC/SMC coculture system, TF activity was higher than that of ECs cultured alone, although the TF activity of the coculture system was still less than that of SMCs cultured alone. However, in later days of coculture, TF activity increased dramatically and was similar to levels found with SMCs cultured alone (Fig. 3).

Activation of NFκB in coculture systems

To determine whether NFκB was activated and associated with TF activity in the coculture system, we stained the coculture for the p65 subunit of NFκB. The coculture was evaluated by confocal microscopy and Z-sections obtained at intervals of 1 μm. The superposition of p65 staining (green color) and nuclear staining (red color) suggested that p65 was localized in the cytoplasm for both SMCs and ECs, and there was no clear evidence of p65 nuclear translocation from

Table 2. Tissue Factor Activity of Endothelial Cell Monolayers Using Different Treatment

Treatments	*Normalized TF activity*
Intact surface	1
Scraping to dislodge ECs	9.6 ± 5.1
Using trypsin to dislodge ECs	3.3 ± 1.8

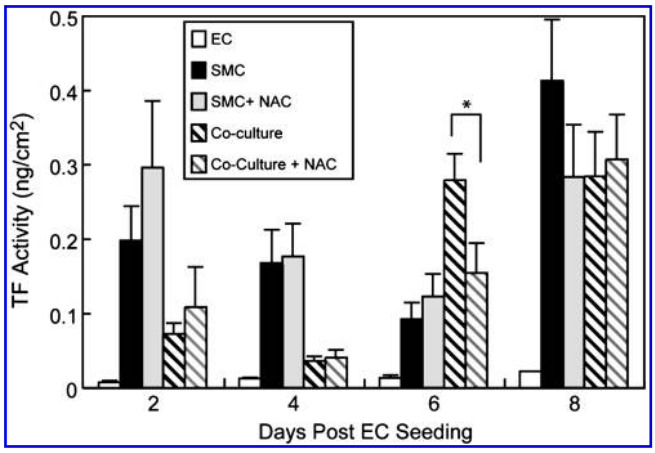

FIG. 3. TF activity in ECs, SMCs, and cocultures. ECs were seeded on day 0 for the coculture, and the activity was measured 2 days after EC seeding. One day after culturing ECs, SMCs, and coculture in the coculture medium with 3.3% serum content, 2 mM NAC was added to the culture medium, and medium with or without NAC was changed every other day. *$p < 0.05$. NAC, N-acetyl-L-cysteine.

days 2 to 9 of coculture. Figure 4A–F shows p65 staining, and nuclear staining was not shown to focus on the distribution of p65 inside cells. On days 4 (Fig. 4A, B) and 9 (Fig. 4C, D) p65 was distributed in the cytoplasm of ECs (Fig. 4B, D) and SMCs (Fig. 4A, C). In contrast, incubation of SMCs (Fig. 4E) and ECs (Fig. 4F) with 100 μM LPS for 4 h before fixation and staining produced nuclear translocation of p65 as evidenced by the bright staining of nuclei compared to low background in cytoplasm. In untreated ECs in monoculture, NFκB was not activated (data not shown). It should be noted that after 9 days of coculture, ECs displayed an apoptotic character, as evidenced by the presence of some vacuoles in NFκB staining (Fig. 4D). The appearance of vacuoles was not evident on day 4 of coculture.

Contraction of EC/SMC coculture system and the coverage of ECs on coculture

Accompanying the dramatic increase in TF activity with duration of the coculture, there was a progressive contraction of the coculture (Fig. 5). We observed formation of clusters of contracted cells, producing numerous cell-free holes between clusters of contracted cells. The contraction of SMCs moved the adherent ECs, as determined by EC-specific 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) staining (data not shown). Using 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (DiI)-labeled Ac-LDL staining of ECs, we observed loss of EC coverage in our coculture system of about 20% after 8 days. Eventually, many isolated clusters of contracted cells were formed and could be observed directly without the aid of a microscope. In some cases, these cells formed a sheet, and peeled off from the surface. Positive staining for EthD-1 concentrated in cell clusters revealed that the cells were apoptotic or necrotic (red color in Fig. 5). Contraction was enhanced when SMCs were cocultured with ECs, suggesting that ECs might modulate SMCs' phenotype toward a differentiated and actively contractile state, as evidenced by the (2–4 days) earlier contraction of the coculture system than SMCs cultured alone. For both SMCs and for coculture, 3.3% serum content was used.

Effect of antioxidant on TF activity and contraction

ROS play an important role in SMC contraction.[28] ROS can increase vascular SMC differentiation, as evidenced by increased level of calponin, SM2 myosin, and alpha-actin.[29] To test this, we incubated the coculture system with the antioxidant NAC at various concentrations (1, 2, and 5 mM) during the coculture period. The effect of NAC on cell contraction is shown in Table 3. At 1 mM, NAC did not affect cell contraction. However, addition of 2 mM NAC and 5 mM NAC increased the time before coculture contraction occurred. However, elevated concentrations of NAC caused cell death. In the control experiments without NAC, ECs and SMCs experienced 4% and 2% cell death, respectively. In the coculture system after 2 days, cell death increased to 7% (Fig. 6). At 5 mM NAC, cell death for ECs cultured alone increased from 4% to 16.7% ($p < 0.01$), and SMCs from 1% to 10% ($p < 0.01$, Fig. 6). In the coculture system, the percentage of dead cells increased from 7% to 11% ($p > 0.1$), suggesting that the interaction of ECs and SMCs helped cells to survive the low ROS level. As 2 mM NAC did not have a significant impact on cell death and delayed cell contraction, this concentration was used in subsequent experiments.

To test whether the increased TF activity was due to elevated levels of ROS, we incubated SMCs and cocultures with NAC 1 day after ECs seeding in the coculture system or after SMCs in the coculture medium (Fig. 3). At days 2 and 4, the addition of NAC had no inhibitory effect on TF activity in SMCs and cocultures. At day 6, when coculture usually contracted in the absence of NAC, the addition of NAC prevented contraction and significantly reduced TF activity in the coculture ($p < 0.05$), suggesting that ROS was not directly involved in TF activity, but indirectly through the regulation of cell contraction.

Discussion

The EC/SMC direct coculture model provides one means to assess cell–cell interactions in an *in vitro* setting. There are several coculture models currently in use: (1) ECs and SMCs grown on opposite sides of membranes or in a Boyden chamber, (2) use of the conditioned medium from one cell with another cell type, and (3) direct coculture of the two cell types. No single system replicates the exact structure of the vessel wall. We chose to work with the direct culture of ECs on SMCs because ECs and SMCs are in close apposition in smaller arteries and arterioles and in tissue-engineered blood vessels. Further, contacts have been observed between ECs and SMCs in this system. Although a mature basement membrane is not present, SMCs secrete a matrix of fibronectin, collagen I, and collagen III that affects EC function.[16] Direct contact also reduces the diffusion distance between the two cell types, which is important for short-lived intermediates such as nitric oxide. Thus, direct EC/SMC coculture provides a useful model of the vessel wall and results can be directly translated to tissue engineering.

TF activity was used as a functional test of EC thrombogenicity because TF is as a key initiator in coagulation cascade. TF and tissue-type plasminogen activator were the only 2 genes that were differentially expressed among 31

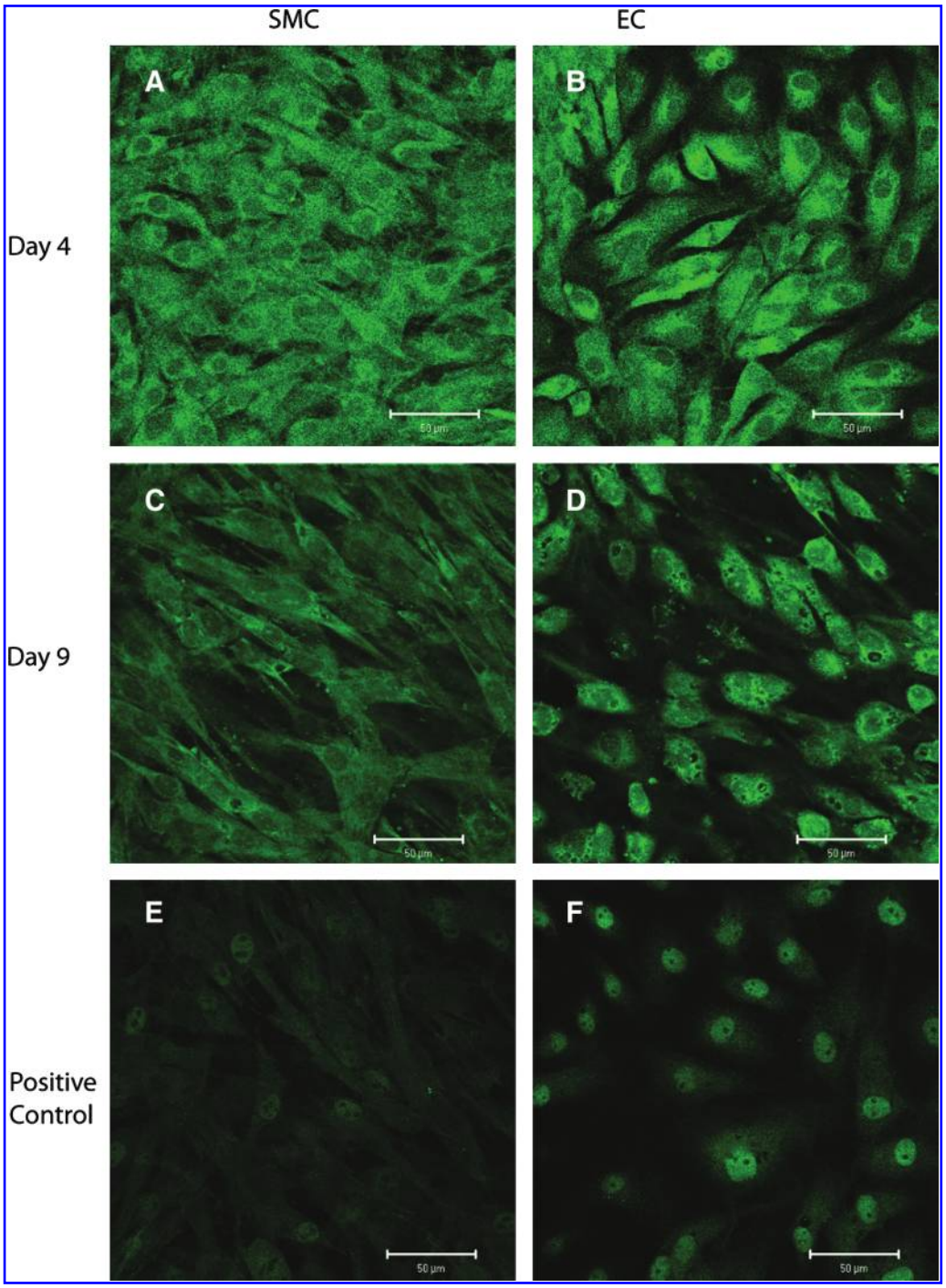

FIG. 4. Immunostaining of p65 in the coculture system. Cocultures were prepared in 3.3% serum as described in Materials and Methods section and stained for the p65 component of NFκB. NFκB is distributed throughout the cytoplasm in SMC and EC (**A–D**). The positive control involved treatment of the cocultures with 100 μM lipopolysaccharide for 4 h. After staining, NFκB is localized in SMC and EC nuclei (**E, F**). NFκB, nuclear factor kappa-B. Color images available online at www.liebertonline.com/ten.

genes of the coagulation system between SMCs from internal mammary artery and saphenous vein, and are known to be involved in thrombus formation and vascular remodeling.[30] Clinically, inhibition of TF activity is an attractive target for the treatment of cardiovascular diseases.[31] Further, the two-step amidolytic assay to monitor factor Xa generation is widely accepted as a functional assay.[18,32,33] Because a controlled system is used for the amidolytic assay, it is much more reproducible than assays that use whole blood or plasma.

Using the direct coculture model, we found that porcine EC/SMC cocultures had lower TF activity than SMC cultured alone, but slightly higher TF activity than ECs cultured alone. Interestingly, TF antigen on ECs cultured alone was

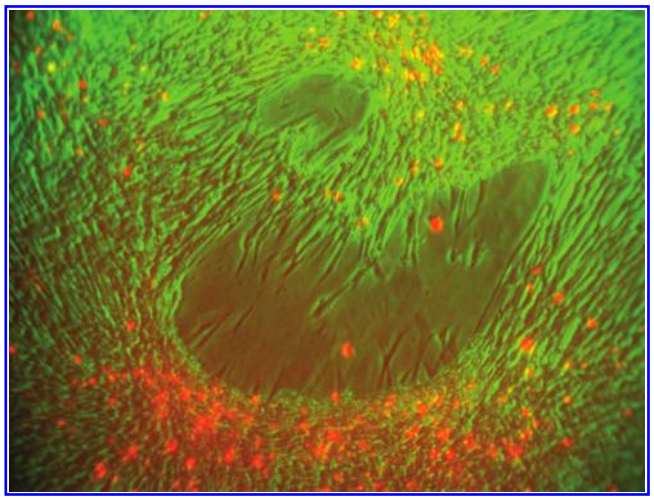

FIG. 5. Contraction of EC/SMC after 8 days of coculture in 3.3% serum. This composite picture consists of the phase contrast picture in green channel, and fluorescent picture of ethidium homodimer-1 staining showed the nuclei of dead cells in red color. The contraction of coculture caused significant cell death in the contracted areas. Color images available online at www.liebertonline.com/ten.

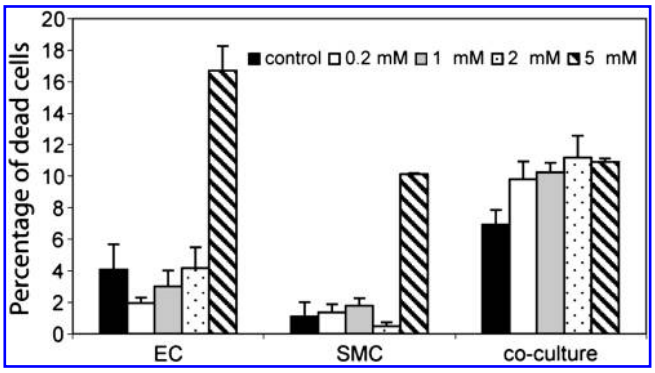

FIG. 6. Effect of NAC on cell death. ECs were seeded on fibronectin-precoated six-well plate and on SMCs that had been in the quiescent medium for 2 days. Cells were incubated with various concentrations of NAC (0, 0.2, 1, 2, and 5 mM) in the coculture medium (3.3% serum content) for another 2 days. LIVE/DEAD cytotoxicity was conducted at the end of experiments.

expressed in a heterogeneous pattern, and TF antigen was detected on some cultured ECs (14%). In coculture, the TF antigen was primarily associated with SMCs. TF activity increased after 5 days of coculture, which corresponded to contraction of EC/SMC cocultures. However, NFκB was not likely involved in the signal transduction pathway, as evidenced by the fact that it was localized in the cytoplasm in an inactive form. Even with 3.3% serum, SMCs still maintained contractile/quiescent phenotype, as evidenced by the elongated morphology (Fig. 4), lack of proliferation, and expression of smooth muscle myosin and calponin.[15] Further, we have no evidence that TF activity increased on ECs in coculture (Table 1).

Correlation between TF antigen expression and TF activity

TF activity is regulated at both transcriptional[21,34–37] and posttranscriptional levels.[38,39] In addition, TF present on the cell surface can bind both factor VII and VIIA, and may not be competent to initiate coagulation, a situation termed "encryption."[40] As a result, immunostaining for TF is not always correlated with TF activity. Noguchi *et al.*[39] found that expression of cell-surface TF apoprotein antigen precedes expression of cell-surface TF coagulant activity by approximately 2 h, which is possibly due to a posttranslational conformational change at the cell surface. In the current study, observation of TF by immunofluorescence may not have identified all of the active TF or reliably discriminated between TF antigen on the apical and basolateral sides of EC. Normally, TF on the basolateral side is shielded from the blood and as such does not normally induce a thrombotic response. Even when the intensity levels of TF staining are the same, TF activity can be strikingly different due to the distribution of TF on the cell membrane, which was observed in our experiments. Nevertheless, we found low TF activity on ECs and a positive correlation between TF antigen expression and TF activity. Namely, EC TF antigen expression and TF activity are both low compared to values for SMCs.

Table 3. Effect of N-Acetyl-L-Cysteine on Cell Contraction

	Contraction time			
Trial	*Control (0 mM)*	*NAC (1 mM)*	*NAC (2 mM)*	*NAC (5 mM)*
1	6 days	N/A	8 days	8 days[a]
2	4 days	4 days	5 days	5 days
3	6 days		7 days	

[a]A small isolated region near the edges of the well contracted on day 6; however, the rest of the well appeared healthy until it fully contracted on day 8.

NAC, N-acetyl-L-cysteine; N/A, not available.

Regulation of TF activity in ECs and SMCs (role of serum, NFκB, and ROS)

Previous studies have shown that in rat aortic SMCs, lysophosphatidic acid, a component that is released by activated platelets and is found in serum, induced TF expression.[20] Fresh 10% fetal calf serum stimulated quiescent human fetal lung fibroblast expression of TF mRNA and procoagulant activity[41] that peaked at 12 h and slowly declined to baseline over the next 36 h. We used 3.3% serum in our studies because ECs would not function well in the serum-free medium. The presence of serum should lead to higher TF activity than would be obtained in the serum-free medium or plasma that should have low levels of lysophosphatidic acid.

In rat SMCs, serum stimulated TF activity through *SP1* binding sites.[42] In porcine aortic ECs, the TF promoter contains one NFκB binding site and two *AP-1* binding sites.[21] Our positive controls using LPS to activate NFκB and to increase TF activity confirmed the role of NFκB in TF activity regulation. However, in the coculture system NFκB was not activated (Fig. 4), suggesting that increased TF activity was not due to NFκB activation.

Recent studies suggest that an increase of ROS promotes a procoagulant state,[13,14,43] through either an NFκB-dependent pathway[44,45] or an NFκB-independent pathway.[38] As TF is a redox-sensitive gene,[46] the accumulation of ROS might lead to increased TF activity. Brisseau *et al.*[38] reported that antioxidants inhibited the LPS-induced TF activity in macrophages, but little change in mRNA expression level was observed, suggesting that the regulation of TF activity by antioxidants could be through a posttranscriptional mechanism. However, in our study, TF activity was not regulated by ROS during the first 4 days of coculture. The inhibition of TF activity by NAC on day 6 was very likely due to the inhibition of SMC contraction, which consequently prevented the exposure of TF on SMCs or the basolateral side of EC monolayer.

During the first few days of coculture, TF activity on cocultured porcine ECs increased compared to ECs cultured alone. This increase was not due to the exposure of TF on SMCs, since in our previous study we showed that EC formed a very tight monolayer during the first 4 days.[15] Therefore, the increased TF activity of coculture was most likely due to the activation of ECs through an NFκB-independent pathway.

Addition of NAC to coculture reduced TF activity; however, NAC also induced cell apoptosis. In the porcine ECs and SMCs, even 5 mM NAC caused significant cell apoptosis ($p < 0.05$, Fig. 6). This effect was also observed in another study in which NAC (10 mM) induced vascular SMC (rat aortic SMC and human aortic SMC) apoptosis,[47] suggesting that a delicate balance of ROS is maintained in cells and/or NAC is toxic to cells.

Contraction of the coculture system and the limitation of two-dimensional culture

In the later days of coculture, we observed a dramatic increase in TF activity, which coincided with the contraction of the coculture. The contraction of SMCs moved the ECs and compromised EC monolayer integrity. Since SMCs express high level of TF on their surfaces and have high level of TF activity than EC, the compromised EC monolayer after contraction is believed to increase TF activity of the coculture. Because accumulated ROS levels were implicated in cell contraction, the incubation of an antioxidant such as NAC would have been expected to repress contraction, which is confirmed in Table 3.

Implication for TEVGs

The seeding of ECs to TEVGs has had only limited functional success. From the current study, we might conclude that the initial seeding might provide an effective layer of nonthrombogenic ECs. However, due to the elevated ROS level and contraction of underlying SMCs, the integrity of EC monolayer was severely compromised, which could lead to thrombosis that is frequently observed in implanted vascular grafts that are composed of ECs and SMCs. To maintain a confluent and functional EC monolayer was challenging in this simplified coculture system. The use of antioxidants might prove promising for maintaining coculture monolayers, but the appropriate concentrations of ROS inhibitors have yet to be determined for the best coculture outcomes.

The ECs that are cultured on quiescent SMCs seem to maintain their functional phenotype, as evidenced by (1) low TF activity for the coculture system before SMC contraction occurs and (2) no NFκB activation observed in ECs before contraction begins. Based on the current study, contraction that exposes SMC TF may increase TF activity, potentially leading to thrombosis.

To avoid direct EC/SMC interaction, HUVECs have been seeded directly to a decellularized matrix.[48] In this case, HUVECs displayed favorable hemocompatible property, as assessed by the expression level of von Willebrand factor, TM, E-selectin, and intercellular adhesion molecule-1.[48] Endothelial seeding on a decellularized matrix is a good alternative to seeding on vascular grafts composed by SMCs. Using a similar coculture system with human cells, Wallace *et al.*[17] found that TF levels in human coculture were less than in monoculture and could be maintained for more than 2 weeks. Further, human ECs did not induce contraction of human SMCs. The discrepancy observed here may be explained by the cell species (human cells vs. porcine cells), since same serum content was used in both studies.

Taken together, our results show that ECs became procoagulant due to the contraction of coculture system that exposed SMCs underneath. Although the exact mechanism needs to be further confirmed, the addition of NAC or other antioxidant seems to be beneficial to inhibit SMC contraction, and maintain a noncoagulant surface. In future studies, the concentration of NAC should to be optimized to avoid the side effect of cell apoptosis.

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Disclosure Statement

L.E.N. has a financial interest in Humacyte, a regenerative medicine company. Humacyte did not fund these studies, and Humacyte did not affect the design, interpretation, or reporting of any of the experiments herein.

References

1. Mitchell, S.L., and Niklason, L.E. Requirements for growing tissue-engineered vascular grafts. Cardiovasc Pathol **12,** 59, 2003.
2. Deutsch, M., Meinhart, J., Fischlein, T., Preiss, P., and Zilla, P. Clinical autologous *in vitro* endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. Surgery **126,** 847, 1999.
3. Ameri, A., Kuppuswamy, M.N., Basu, S., and Bajaj, S.P. Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. Blood **79,** 3219, 1992.
4. Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, E.S., McCrae, K.R., Hug, B.A., Schmidt, A.M., and Stern, D.M. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood **91,** 3527, 1998.
5. Hedeman Joosten, P.P., Verhagen, H.J., Heijnen-Snyder, G.J., Sixma, J.J., de Groot, P.G., Eikelboom, B.C., and Elisen, M.G. Thrombomodulin activity of fat-derived microvascular

endothelial cells seeded on expanded polytetrafluorethylene. J Vasc Res **36,** 91, 1999.
6. Gillis-Haegerstrand, C., Frebelius, S., Haegerstrand, A., and Swedenborg, J. Cultured human endothelial cells seeded on expanded polytetrafluoroethylene support thrombin-mediated activation of protein C. J Vasc Surg **24,** 226, 1996.
7. Sapienza, P., di Marzo, L., Cucina, A., Corvino, V., Mingoli, A., Giustiniani, Q., Ziparo, E., and Cavallaro, A. Release of PDGF-BB and bFGF by human endothelial cells seeded on expanded polytetrafluoroethylene vascular grafts. J Surg Res **75,** 24, 1998.
8. Tunstall, A., Eberhart, R.C., and Prager, M.D. Endothelial cells on Dacron vascular prostheses: adherence, growth, and susceptibility to neutrophils. J Biomed Mater Res **29,** 1193, 1995.
9. Cenni, E., Granchi, D., Ciapetti, G., Verri, E., Cavedagna, D., Gamberini, S., Cervellati, M., Di Leo, A., and Pizzoferrato, A. Expression of adhesion molecules on endothelial cells after contact with knitted Dacron. Biomaterials **18,** 489, 1997.
10. Remy, M., Bareille, R., Villars, F., Rouais, F., Gorodkov, A., and Bordenave, L. Endothelial cells lining polyester fabric express pro-coagulant phenotype *in vitro*. Med Biol Eng Comput **36,** 256, 1998.
11. Nemerson, Y. Tissue factor and hemostasis. Blood **71,** 1, 1988.
12. Pendurthi, U.R., Williams, J.T., and Rao, L.V. Inhibition of tissue factor gene activation in cultured endothelial cells by curcumin. Suppression of activation of transcription factors Egr-1, AP-1, and NF-kappa B. Arterioscler Thromb Vasc Biol **17,** 3406, 1997.
13. Griendling, K.K., and Alexander, R.W. Oxidative stress and cardiovascular disease. Circulation **96,** 3264, 1997.
14. Taniyama, Y., and Griendling, K.K. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. Hypertension **42,** 1075, 2003.
15. Lavender, M.D., Pang, Z., Wallace, C.S., Niklason, L.E., and Truskey, G.A. A system for the direct co-culture of endothelium on smooth muscle cells. Biomaterials **26,** 4642, 2005.
16. Wallace, C.S., Strike, S.A., and Truskey, G.A. Smooth muscle cell rigidity and extracellular matrix organization influence endothelial cell spreading and adhesion formation in coculture. Am J Physiol Heart Circ Physiol **293,** H1978, 2007.
17. Wallace, C.S., Champion, J.C., and Truskey, G.A. Adhesion and function of human endothelial cells co-cultured on smooth muscle cells. Ann Biomed Eng **35,** 375, 2007.
18. Lin, M.C., Almus-Jacobs, F., Chen, H.H., Parry, G.C., Mackman, N., Shyy, J.Y., and Chien, S. Shear stress induction of the tissue factor gene. J Clin Invest **99,** 737, 1997.
19. Schecter, A.D., Giesen, P.L., Taby, O., Rosenfield, C.L., Rossikhina, M., Fyfe, B.S., Kohtz, D.S., Fallon, J.T., Nemerson, Y., and Taubman, M.B. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. J Clin Invest **100,** 2276, 1997.
20. Cui, M.Z., Zhao, G., Winokur, A.L., Laag, E., Bydash, J.R., Penn, M.S., Chisolm, G.M., and Xu, X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. Arterioscler Thromb Vasc Biol **23,** 224, 2003.
21. Moll, T., Czyz, M., Holzmuller, H., Hofer-Warbinek, R., Wagner, E., Winkler, H., Bach, F.H., and Hofer, E. Regulation of the tissue factor promoter in endothelial cells. Binding of NF kappa B-, AP-1-, and Sp1-like transcription factors. J Biol Chem **270,** 3849, 1995.
22. Mulder, A.B., Hegge-Paping, K.S., Magielse, C.P., Blom, N.R., Smit, J.W., van der Meer, J., Hallie, M.R., and Bom, V.J. Tumor necrosis factor alpha-induced endothelial tissue factor is located on the cell surface rather than in the subendothelial matrix. Blood **84,** 1559, 1994.
23. Mechtcheriakova, D., Wlachos, A., Holzmuller, H., Binder, B.R., and Hofer, E. Vascular endothelial cell growth factor-induced tissue factor expression in endothelial cells is mediated by EGR-1. Blood **93,** 3811, 1999.
24. Colucci, M., Balconi, G., Lorenzet, R., Pietra, A., Locati, D., Donati, M.B., and Semeraro, N. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest **71,** 1893, 1983.
25. Ryan, J., Brett, J., Tijburg, P., Bach, R.R., Kisiel, W., and Stern, D. Tumor necrosis factor-induced endothelial tissue factor is associated with subendothelial matrix vesicles but is not expressed on the apical surface. Blood **80,** 966, 1992.
26. Drake, T.A., Morrissey, J.H., and Edgington, T.S. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. Am J Pathol **134,** 1087, 1989.
27. Mulder, A.B., Blom, N.R., Smit, J.W., Ruiters, M.H., van der Meer, J., Halie, M.R., and Bom, V.J. Basal tissue factor expression in endothelial cell cultures is caused by contaminating smooth muscle cells. Reduction by using chymotrypsin instead of collagenase. Thromb Res **80,** 399, 1995.
28. Rhoades, R.A., Packer, C.S., Roepke, D.A., Jin, N., and Meiss, R.A. Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. Can J Physiol Pharmacol **68,** 1581, 1990.
29. Su, B., Mitra, S., Gregg, H., Flavahan, S., Chotani, M.A., Clark, K.R., Goldschmidt-Clermont, P.J., and Flavahan, N.A. Redox regulation of vascular smooth muscle cell differentiation. Circ Res **89,** 39, 2001.
30. Payeli, S.K., Latini, R., Gebhard, C., Patrignani, A., Wagner, U., Luscher, T.F., and Tanner, F.C. Prothrombotic gene expression profile in vascular smooth muscle cells of human saphenous vein, but not internal mammary artery. Arterioscler Thromb Vasc Biol **28,** 705, 2008.
31. Steffel, J., Luscher, T.F., and Tanner, F.C. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation **113,** 722, 2006.
32. Camera, M., Giesen, P.L., Fallon, J., Aufiero, B.M., Taubman, M., Tremoli, E., and Nemerson, Y. Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. Arterioscler Thromb Vasc Biol **19,** 531, 1999.
33. Cavusoglu, E., Chen, I., Rappaport, J., and Marmur, J.D. Inhibition of tissue factor gene induction and activity using a hairpin ribozyme. Circulation **105,** 2282, 2002.
34. Mackman, N. Regulation of the tissue factor gene. Thromb Haemost **78,** 747, 1997.
35. Shi, R.J., Simpson-Haidaris, P.J., Lerner, N.B., Marder, V.J., Silverman, D.J., and Sporn, L.A. Transcriptional regulation of endothelial cell tissue factor expression during *Rickettsia rickettsii* infection: involvement of the transcription factor NF-kappaB. Infect Immun **66,** 1070, 1998.
36. Crossman, D.C., Carr, D.P., Tuddenham, E.G., Pearson, J.D., and McVey, J.H. The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. J Biol Chem **265,** 9782, 1990.
37. Parry, G.C., and Mackman, N. Transcriptional regulation of tissue factor expression in human endothelial cells. Arterioscler Thromb Vasc Biol **15,** 612, 1995.

38. Brisseau, G.F., Dackiw, A.P., Cheung, P.Y., Christie, N., and Rotstein, O.D. Posttranscriptional regulation of macrophage tissue factor expression by antioxidants. Blood **85,** 1025, 1995.
39. Noguchi, M., Sakai, T., and Kisiel, W. Correlation between antigenic and functional expression of tissue factor on the surface of cultured human endothelial cells following stimulation by lipopolysaccharide endotoxin. Thromb Res **55,** 87, 1989.
40. Bach, R., and Rifkin, D.B. Expression of tissue factor procoagulant activity: regulation by cytosolic calcium. Proc Natl Acad Sci U S A **87,** 6995, 1990.
41. Bloem, L.J., Chen, L., Konigsberg, W.H., and Bach, R. Serum stimulation of quiescent human fibroblasts induces the synthesis of tissue factor mRNA followed by the appearance of tissue factor antigen and procoagulant activity. J Cell Physiol **139,** 418, 1989.
42. Taby, O., Rosenfield, C.L., Bogdanov, V., Nemerson, Y., and Taubman, M.B. Cloning of the rat tissue factor cDNA and promoter: identification of a serum-response region. Thromb Haemost **76,** 697, 1996.
43. Ruef, J., Peter, K., Nordt, T.K., Runge, M.S., Kubler, W., and Bode, C. Oxidative stress and atherosclerosis: its relationship to growth factors, thrombus formation and therapeutic approaches. Thromb Haemost **82 Suppl 1,** 32, 1999.
44. Schreck, R., Rieber, P., and Baeuerle, P.A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J **10,** 2247, 1991.
45. Manna, S.K., Zhang, H.J., Yan, T., Oberley, L.W., and Aggarwal, B.B. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J Biol Chem **273,** 13245, 1998.
46. Inoue, N., Ramasamy, S., Fukai, T., Nerem, R.M., and Harrison, D.G. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. Circ Res **79,** 32, 1996.
47. Chiu, J.J., Chen, L.J., Chang, S.F., Lee, P.L., Lee, C.I., Tsai, M.C., Lee, D.Y., Hsieh, H.P., Usami, S., and Chien, S. Shear stress inhibits smooth muscle cell-induced inflammatory gene expression in endothelial cells: role of NF-{kappa}B. Arterioscler Thromb Vasc Biol **25,** 963, 2005.
48. Remy-Zolghadri, M., Laganiere, J., Oligny, J.F., Germain, L., and Auger, F.A. Endothelium properties of a tissue-engineered blood vessel for small-diameter vascular reconstruction. J Vasc Surg **39,** 613, 2004.

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