Angiogenic Microvascular Endothelial Cells Release Microparticles Rich in Tissue Factor That Promotes Postischemic Collateral Vessel Formation

Gemma Arderiu, Esther Peña, Lina Badimon

Objective—Therapeutic angiogenesis is a promising strategy for treating ischemia. Our previous work showed that endogenous endothelial tissue factor (TF) expression induces intracrine signaling and switches-on angiogenesis in microvascular endothelial cells (mECs). We have hypothesized that activated mECs could exert a further paracrine regulation through the release of TF-rich microvascular endothelial microparticles (mEMPs) and induce neovascularization of ischemic tissues.

Approach and Results—Here, we describe for the first time that activated mECs are able to induce reparative neovascularization in ischemic zones by releasing TF-rich microparticles. We show in vitro and in vivo that mEMPs released by both wild-type and TF-upregulated-mECs induce angiogenesis and collateral vessel formation, whereas TF-poor mEMPs derived from TF-silenced mECs are not able to trigger angiogenesis. Isolated TF-bearing mEMPs delivered to nonperfused adductor muscles in a murine hindlimb ischemia model enhance collateral flow and capillary formation evidenced by MRI. TF-bearing mEMPs increase angiogenesis operating via paracrine regulation of neighboring endothelial cells, signaling through the β1-integrin pathway Rac1–ERK1/2–ETS1 and triggering CCL2 (chemokine [C-C motif] ligand 2) production to form new and competent mature neovessels.

Conclusions—These findings demonstrate that TF-rich mEMPs released by microvascular endothelial cells can overcome the consequences of arterial occlusion and tissue ischemia by promoting postischemic neovascularization and tissue reperfusion. (Arterioscler Thromb Vasc Biol. 2015;35:348-357. DOI: 10.1161/ATVBAHA.114.303927.)

Key Words: angiogenesis-inducing agents ■ cell-derived microparticles■ endothelial cells ■ hindlimb ischemia ■ thromboplastin

Angiogenesis plays a key role in physiological processes, including embryonic development and wound repair, as well as in various pathologies such as ischemic diseases, cancer, diabetic retinopathy, or chronic inflammation including atherosclerosis.1 Angiogenesis is an active process that depends on a delicate interplay between endothelial cells (ECs) and smooth muscle cells (SMCs).2 Modulation of angiogenesis represents a potential therapeutic tool against a large number of diseases. An increased neovascularization leading to wound healing and ischemic injury repair is a much searched therapeutic goal. Recent studies support the concept of a proangiogenic effect of microparticles, depending on their composition and concentration.3,4 High amounts of microparticles were detected in mouse after hindlimb ischemia, and their increased levels were associated to restoring the consequences of arterial occlusion and tissue ischemia by promoting postischemic neovascularization.8

Microparticles are 0.1- to 1-μm plasma membrane–derived vesicles shed from stimulated cells.9 ECs can release microparticles when they are activated. Endothelial-derived microparticles (EMPs) are subcellular fragments of the EC lipid bilayer. EMPs that are release in relatively low numbers in physiological conditions are increasingly released on cell activation.10–12 EMPs harbor membrane proteins and intracellular components involved in cell signaling that are characteristic of the original parent cell. Microparticles from activated EC cells are known to carry tissue factor (TF), and their procoagulant properties during disease have been characterized.13-15

TF is a low-molecular weight glycoprotein that initiates the extrinsic clotting cascade and is considered a major regulator of arterial thrombogenicity.16,17 In addition, during the past decades, TF has also shown to be involved in intracellular signaling pathways in cell function regulation (ie, proliferation,18 migration,19,20 gene expression,21 and mature microvessel formation).22,23 Our previous work showed that proangiogenic microvascular ECs (mECs) regulate intracellular TF expression and by intracrine AKT-ETS signaling induce chemokine (C-C motif) ligand 2 (CCL2) expression and secretion to recruit SMC toward the mECs to form mature neovessels.21,24

Received on: February 3, 2014; final version accepted on: September 26, 2014.

From the Cardiovascular Research Center (CSIC-ICCC), IIB–Sant Pau and Hospital de Sant Pau, Barcelona, Spain (G.A., E.P., L.B.); and Cardiovascular Research Chair Universitat Autònoma de Barcelona, Barcelona, Spain (L.B.).

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303927/-/DC1. Correspondence to Lina Badimon, PhD, Cardiovascular Research Center, CSant Antoni M’Claret 167, 08025 Barcelona, Spain. E-mail lbadimon@csic-iccc.org

© 2014 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.114.303927

348
TF is found in 2 different molecular forms: full-length TF (fTF), an integral membrane protein; and alternatively spliced TF, a protein that lacks a transmembrane domain and is secreted in a soluble form.25 Both forms have been associated with the angiogenic processes. However, TF can also be released from the cell membrane in the form of microparticles, which constitute a pool of circulating TF.26,27 Increased number of TF-bearing microparticles resulting from vesicle release from platelets and other blood cells or tumor cells and their procoagulant activity has been extensively documented in several vascular diseases.28–30

Despite a growing interest in MPs as inducers of angiogenesis, little is known on the effects of TF-bearing mECs-derived microparticles (mEMPs) on ischemic tissue reperfusion. We hypothesized that phenotypic changes in mECs may induce release of mEMPs with functional angiogenic activity in ischemic tissue reperfusion. Our data show that TF released from activated mECs in the form of microparticles interact via paracrine signaling with other mECs operating through a β1-integrin pathway, TF-bearing mEMPs (exogenous TF) signals through β1-integrin engagement.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Microvascular EC Forming Tube-Like Structures Release TF-Bearing Endothelial Microparticles

In contrast to monocytes or tumor cells, mECs do not constitutively express TF and release microparticles spontaneously; however, after stimulation, TF may be released from cells in form of microparticles. First, we studied whether during tube-like formation, activated mECs release TF-rich microparticles. Results showed that, in quiescent mECs (resting cells in culture), intracellular TF levels were very low and localized around the cell nucleus (Figure 1A). However, when mECs were stimulated to migrate by scratch lesions in cell monolayers, TF expression increased at border edges of cells and in cell lamellipodia in the cell migratory front. Living cell membrane dye and confocal microscopy analysis revealed that angi-tube-forming cells spontaneously shed membrane vesicles from the cell plasma membrane. Formed and released vesicles had a roughly spherical shape and varying size from 0.5 to 1 μm (Figure 1B). To study whether these vesicles contain TF, we analyzed the endothelial conditioned media of human dermal endothelial cell and human microvascular endothelial cell-1 either in quiescent state, in migrating phenotype, or in forming tube-like structures phenotype (cultured on matrigel for 18 hours). As shown in Figure 1C, the number of mEMPs positive for TF analyzed by flow cytometry was much higher in the endothelial conditioned media of angiobule-forming cells than in the endothelial conditioned media of quiescent cells. Similar results were obtained using human microvascular endothelial cell-1 (Figure IIA in the online-only Data Supplement).

These results show that although release of TF-bearing mEMPs is limited in static quiescent mECs, it is highly activated in angiobule-forming mECs.

Effect of TF-Bearing mEMPs in Microvascular EC Tube-Like Formation

Previously, we demonstrated that in mECs endogenous TF contributes to angiogenic processes.25 Here, we investigated whether the released TF-rich mEMPs were able to induce endothelial angiobule formation. TF-rich microparticles (TF+mEMPs) were obtained from migrating TF overexpressing cells to have high

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Microvascular endothelial cell microparticle (mEMP) shedding. A, Immunofluorescence staining of quiescent or migrating mECs, staining for tissue factor (TF; green), actin (red), and nuclear staining (blue). B, Confocal micrograph of living cell stained mECs cultured in 3-dimensional basement membrane–forming angiotubes and showing vesicle release (red). C, Flow cytometry analysis of supernatants from quiescent, migrating or cells forming tube-like structures. Results are expressed as mEMPs per mL corresponding to total number of events, positive annexin V, or double-positive for annexin V and TF; data are shown as means±SEM of 3 independent experiments. Statistical analysis was performed by ANOVA followed by Tukey post hoc test (P<0.05, **P<0.01, and ***P<0.001).
Figure 2. Tissue factor (TF)+microvascular endothelial cell microparticles (mEMPs) activate β1-integrin signaling. A, Phase-contrast micrographs showing the morphology of mECs treated with scrambled (human dermal endothelial cell [HDMEC]) or TF siRNA (HDMEC-TF−) in the presence or in the absence of TF+mEMPs or TF-EMPs, 18 hours after culturing on 3-dimensional basement membrane (3DBM) cultures. B, Average of tube % area covered, branching points, and total length were measured in 5 randomly selected areas from 3 independent experiments. Values are ±SEM. Statistical analysis was performed by ANOVA followed by Tukey post hoc test (*P<0.05 and **P<0.01). C, Immunofluorescence staining of mECs (red) after 18 hours of 3DBM cultures in the presence of TF+mEMPs (green). D, A maximal projection was created from a spatial data set acquired every 0.1 μm (vertical thickness) in xzy scanning mode, and thereafter a 3D projection was performed creating a projection every 2 grades. E, Flow cytometry analysis of TF+mEMPs adhered onto mECs or TF+mEMPs adhered onto mECs previously incubated with anti–β1-integrin–blocking antibody. Results are expressed as % of mECs positive for green fluorescent protein (GFP) (TF+EMPs); data are shown as mean±SEM of 3 independent experiments. ***P<0.001. F, mECs adhered to collagen-coated wells or TF-EMPs-coated wells; mECs preincubated with specific β1-integrin–blocking antibody adhered to TF-EMPs-coated wells. Flattened cells were counted; data are shown as mean±SEM of 3 independent experiments. ***P<0.001. G, β1-integrin regulates signaling induced by TF+mEMPs in mECs cultured for 18 hours in 3DBM. TF+mEMPs signal through Rac1, ERK1/2 and ETS1 activation, and chemokine (C-C motif) ligand 2 (CCL2) protein. Western blots are representative of three independent experiments. H, CCL2 concentration in media from HMEC-1 in different conditions cultured in 3DBM for 18 hours, values represent mean±SD from 3 different experiments. Statistical analysis was performed by Student t test, ***P<0.001.
amounts of TF (Figure IIB in the online-only Data Supplement). Cells were cultured on 3-dimensional (3D) basement membrane and incubated with TF+mEMPs (3.5×10⁷ mEMPs containing 100 pmol/L of TF). We observed that TF+mEMPs induced a complex network of angiotubes and with many branching points (Figure 2A and 2B). This effect was not observed in mECs exposed to TF-poor mEPs (TF−mEMPs; 3.5×10⁷ mEMPs containing <2 pmol/L of TF). TF−mEMPs were obtained from TF-silenced mECs that were forced to migrate for 48 hours to stimulate microparticles release. Silencing TF gene (TF siRNA) induced inhibition of angiotope formation that was partially rescued by addition of TF+mEMPs, but not by the addition of TF−mEMPs. Control cells were always treated with random siRNA as a proper control. TF-silenced cells released negligible amounts of mEMPs positive for TF, which by flow cytometry were positive for annexin V but negative for TF (Figure IIC in the online-only Data Supplement).

Moreover, confocal microscopy of mECs forming tube-like structures (red) and labeled TF+mEMPs (green) showed that TF+mEMPs are bound to the mEC surface (Figure 2C and 2D).

TF+mEMPs Ligate β1-Integrin on mECs Surfaces and Induce Signaling

Because exogenous TF has been shown to interact with integrins,31 and it has been demonstrated that microparticle-TF induce EC proliferation by β1-integrin interaction,32 the possibility that TF+mEMPs-induced angiotope formation was mediated by the interaction with β1-integrin in mECs was next examined. By flow cytometry, we studied whether TF+mEMPs to ligate mECs through β1-integrin. Microvascular ECs pretreated with β1-integrin–blocking antibody were treated with TF+mEMPs, and the number of cells positive for GFP (green fluorescent protein)-TF+mEMPs was analyzed. Pretreatment of mECs with the β1-integrin–blocking antibody inhibited TF+mEMPs binding to mECs (Figure 2E). These results were corroborated by adhesion studies, in which tissue culture plates were coated with collagen 1%, as positive controls, or TF−mEMPs after which mECs were seeded. Figure 2F shows that the mECs bound to TF+mEMP-coated plates in a β1-integrin–dependent fashion because β1-blocking antibodies significantly reduced adhesion.

Incubation of mECs with TF+mEMPs increased TF protein levels in mEC-forming tubes; however, previous incubation of mECs with β1-integrin–blocking antibody suppressed the increased TF levels (Figure 2G). Increased TF levels were because of TF transfer from TF+mEMPs (Figure III in the online-only Data Supplement).

Previously, we had demonstrated that TF-intracrine signaling in mECs induces tube formation and stabilization through AKT-ETS1 activation and CCL2 expression.23 Here, we investigated whether the TF transported by mEMPs and ligated to mECs through integrins was able to induce similar signaling. Result showed that TF from TF+mEMPs was able to trigger signaling through Rac1, ERK1/2, ETS1 activation, and finally increase CCL2 protein levels (Figure 2G). To confirm these results and because CCL2 is a secreted protein, we analyzed CCL2 concentration in mEC culture supernatants. CCL2 concentration was significantly increased in mECs exposed to TF+mEMPs (825.3±57 versus 467.25±12.6 pg/mL without TF+mEMPs). In agreement with the Western blot results, CCL2 secreted levels were significantly reduced in cells preincubated with β1-integrin–blocking antibody (392±23 pg/mL; Figure 2H). As shown in Figure IV in the online-only Data Supplement, TF+mEMPs induce β1-integrin mRNA expression in mECs (>50%). Even in...
β1-integrin–silenced mECs, the exposure of mECs to TF+mEMPs induces de novo synthesis of β1-integrin mRNA. These results indicate that extracellular TF needs β1-integrin engagement before triggering signaling, while as we showed before endogenous TF signals via intracrine pathways through AKT.

To exclude any possible thrombin effects in the process, we treated cells with hirudin. Results show that CCL2 expression was unaffected (Figure V in the online-only Data Supplement).

To investigate whether PAR2 was involved in TF+mEMP-induced signaling, we silenced PAR2 in mECs. In PAR2-silenced cells, TF+mEMPs triggered signaling was unaffected (Figure VI in the online-only Data Supplement). These results indicate that interaction between mECs and TF+mEMP does not require PAR2.

TF+mEMPs Induce Vessel Growth

Ex Vivo and In Vivo

An ex vivo aortic sprouting model was used to test the angiogenic effects of TF transported in mEMPs. Segments of aortas isolated from nude mice were implanted in matrigel plates containing: control buffer, vascular endothelial growth factor/fibroblast growth factor 2 (VEGF/bFGF), TF+mEMPs, and recombinant TF protein, as a positive control. Figure 3 shows that VEGF/bFGF and TF+mEMPs enhanced sprouting when compared with basal levels. Similar results were obtained with recombinant TF protein that also contains fTF (Figure VII in the online-only Data Supplement). The blockade of β1-integrin with the functional β1-blocking antibody

Figure 4. Tissue factor (TF)+microvascular endothelial cell microparticles (mEMPs) induce angiogenesis in vivo. A, Top, A macroscopic view of representative skin tissue 7 days after injection of matrigel plugs. Bottom, Immunofluorescence staining for von Willebrand factor–positive cells. Matrigel plugs contain control solvent (PBS); vascular endothelial growth factor/fibroblast growth factor 2 (VEGF/bFGF) (50 ng/mL each); TF+mEMPs (100 pmol/L); or recombinant TF (100 pmol/L).

B, Quantification of tube % covered area, branch points, and number of vessels per field. Values represent mean±SD from 6 animals for each group. Statistical analysis was performed by ANOVA followed by Tukey post hoc test (***P<0.001 vs control cells).

C, Western blots show TF, β1-integrin, Rac1, phospho-ERK1/2, ERK1/2, p-ETS1, ETS1, and chemokine (C-C motif) ligand 2 (CCL2) protein levels in skin tissue. To test the equal loading, the Western blots were reproved for β-actin. Histogram shows densitometry analysis of the average levels for total protein to β-actin; results are expressed as ±SEM. Statistical analysis was performed by ANOVA followed by Tukey post hoc test, **P<0.01 and ***P<0.001 vs control skin tissue and #P<0.01 and ###P<0.001 vs vascular endothelial growth factor/fibroblast growth factor 2 (VEGF/bFGF).
resulted in abrogation of vessel outgrowth. Thus, exogenous TF requires β1-integrin ligation to trigger sprouting. In a second experimental approach, we examined whether TF+mEMPs could induce angiogenesis in vivo. Matrigel plugs were subcutaneously injected in nude mice. Plugs contained control buffer, VEGF/bFGF, TF+mEMPs, and recombinant TF protein. As shown in Figure 4A, top, when we injected a matrigel plug with control buffer, little vascularization was found in the skin around the plug. However, when we injected a plug with VEGF/bFGF, tissue surrounding the matrigel plug implants showed well-developed zones of neovascularization. This neovascularization effect in the tissue around the plug was better developed in plugs with TF+mEMPs, showing thick main vessels, with many branches that generated secondary branches. Similar results were observed when recombinant TF protein was added in the matrigel. Quantification of area covered by capillaries and branching points (Figure 4B) gave highly significant increases in TF+mEMP-exposed animals. These results were confirmed by histology and blood vessels observation by von Willebrand factor–positive staining in the skin around to the plug (Figure 4A, bottom). As shown in Figure VIII in the online-only Data Supplement, effects of TF+mEMPs were not mediated by VEGF/VEGFR. To evidence the role of TF in this new blood vessel formation, we determined TF, β1-integrin protein, Rac1, ERK1/2, and ETS1 protein levels in skin surrounding the plug (Figure 4C). Western blots show that TF was significantly increased in all situations except in control skin. Moreover, Figure IX in the online-only Data Supplement shows that skin tissue with recombinant TF- and VEGF-matrigel plugs shows soluble TF (rTF) protein expression, whereas skin tissue with TF+mEMPs-matrigel plugs only show fTF protein expression. TF expression was also accompanied by β1-integrin, Rac1 increases and ERK1/2 and ETS1 activation, with final increases in CCL2 protein levels (Figure 4C). Thrombin effect was also evaluated and excluded (Figure X in the online-only Data Supplement). These results indicate that TF+mEMPs are able to induce angiogenesis in vivo, with a significantly higher effect than VEGF/bFGF.

Local Administration of TF+mEMPs Stimulates Angiogenesis in Ischemic Mice Hind Limbs

The highly significant effects of TF+mEMPs in vitro and in vivo prompted us to investigate in a therapeutic fashion whether TF+mEMPs could induce ischemic tissue reperfusion. We studied the effects of TF+mEMPs administration on neovessel formation in ischemic hind limbs after femoral ligation in nude mice. One day after surgery (ligation of arteries), we injected PBS; 100 pmol/L recombinant TF protein, 100 pmol/L TF+mEMPs, 1×10⁷ TF overexpressing human microvascular endothelial cell-1 cells, or 1×10⁷ TF-silenced human microvascular endothelial cell-1 cells. Injections were administrated into 4 delivery sites in the ischemic area (25 μL for each site total volume of 100 μL). Although 16% of animals in the control group (PBS injection) had a Qi scale of 4, in the TF+mEMP-treated group, no animal had ambulatory impairment. Macroscopic observation of mouse hind limbs at day 21 after ligation showed that TF+mEMPs were able to induce new vessel formation because new capillaries could be observed in the ischemic zone when compared with animals treated only with control buffer (Figure 5A, top). To evaluate the appearance of mature complex new blood vessels, α-actin immunostaining for detecting microvessels with pericytes was performed. Confocal analysis demonstrated that TF+mEMPs significantly increased collateral capillary formation in hindlimb ischemia on day 21 (Figure 5A, bottom). Finally, protein analysis revealed an activation of the TF signaling pathway within the ischemic hindlimb in the TF+mEMPs injection group (Figure 5C). Aduction muscle tissue from the enriched capillary zone was processed by Western blot. Administration of TF+mEMPs significantly promoted activation of TF signaling. The newly formed capillary area had upregulated β1-integrin, Rac1 levels, and ERK1/2 and ETS1 activation and finally CCL2 expression. Because TF-silenced cells are not able to generate TF-bearing mEMPs (Figure IIC in the online-only Data Supplement), capillary formation and pathway activation were not affected by the administration of ECs TF− (Figure 5).

Magnetic resonance angiography that measures patent functional vessels (with flowing blood) was then used to visualize the induced therapeutic angiogenesis and collateral vessel formation after hindlimb ischemia. Using 2D-time-of-flight angiography, we observed that the number of visible collateral vessels was highly increased in animals treated with TF+mEMPs compared with untreated animals or those treated with mEMPs not positive for TF (TF EMPS) after surgery (Figure 6A). Computer-assisted measurements of cross-sectional area of perfused vasculature (Figure 6B) were made on transverse images from the 2D-time-of-flight angiography data sets using thresholding. After 21 days of total ligation (20 days of treatment), the mean total cross-sectional area of perfused vasculature in the ligated limb was 2.5-fold higher in TF+mEMPs than that of the PBS or TF-EMP-treated group (Figure 6C).

Together, these results indicated that the administration of TF+mEMPs induce signaling pathways that contributed to postischemic hindlimb neovascularization and collateral competent vessel formation. TF+mEMPs bind to β1-integrin that signals through Rac1, ERK1/2, and ETS1-producing CCL2 that then recruits pericytes toward ECs forming mature-stable neovessels with flowing blood.

Animals were tested for tentative activation of systemic coagulation but alterations were not evidenced. Clotting time-intrinsic coagulation pathway was 61±3 s in animals treated with TF+mEMPs versus 58±6 s in control animals, and clotting time-extrinsic coagulation pathway was 223±8 s in animals treated with TF+mEMPs versus 229±7.5 s in control animals.

Discussion

TF has now a well-recognized role because a signaling molecule involved in the regulation of angiogenesis. Different studies have demonstrated the ability of TF to interact with cell surface integrins, inducing cell signaling pathway activation. This is the first report on the direct action of TF transported in microparticles derived from microvascular endothelium on angiogenesis, and in collateral vessel formation in ischemic limbs. Our data indicate that TF released by activated mECs in the form of microparticles is able to adhere to other mECs in a β1-dependent fashion and increase endothelial tube-like formation.
through β1-integrin, Rac1, ERK1/2, and ETS1 activation and finally CCL2 expression. In a hindlimb ischemia model, the administration of TF+mEMPs are able to increase functional new vessels and collaterals in vivo.

Depending on the cell type, cell surface composition, and concentration, microparticles have been described with capacity to increase or decrease angiogenesis.5,36,37 Whether mECs are able to release TF-bearing mEMPs may be dependent on the trigger of mEC activation. In our study, we have demonstrated that the mECs activated to induce angi tube formation release microparticles that are rich in TF, which further enforce neovessel formation. These results indicated

Figure 5. Delivery of tissue factor (TF)+microvascular endothelial cell microparticles (mEMPs) promotes collateral capillary formation and angiogenesis in vivo. Animals 24 hours after ischemic hind limbs femoral arterectomy were injected with 100 μL of PBS (hindlimb ischemia [HLI]); recombinant TF protein (100 pmol/L); TF+mEMPs (100 pmol/L); TF overexpressing endothelial cells (ECs; 1×10⁷); or TF-silenced ECs (1×10⁷). Injections were made into 4 delivery sites in the ischemic area (25 μL for each site). A, Top, Macroscopic appearance of mouse hindlimb 21 days after surgical excision on the left femoral artery. Bottom, Immunofluorescence staining of frozen sections of ischemic adductors with antibodies against α-actin (green) and nuclear staining (blue). Images are representative from 6 animals each group. B, Quantification of number of vessels per field. Statistical analysis was performed by ANOVA followed by Tukey post hoc test, *P<0.01 and **P<0.001 vs HLI with PBS; and #P<0.01 and ##P<0.001 vs HLI + ECs TF−. C, Western blots show TF, β1-integrin, Rac1, p-ERK1/2, ERK1/2 p-ETS1, ETS1, and chemokine (C-C motif) ligand 2 (CCL2) protein levels in adductor muscle. To test the equal loading, the Western blots were reproved for β-actin.
Neovascularization can be considered as the culmination of several steps: first, degradation of extracellular matrix, migration, adhesion, and proliferation of ECs; second, formation of a new 3D tube, which then lengthens from its tip and circulation is re-established; and finally, recruitment of SMCs that must migrate as well, and adhere to the newly formed EC capillaries. The initial stimulus that leads SMCs to migrate is not well-understood. Our previous work showed that microvascular endothelial TF induces CCL2 expression and secretion that recruit SMC toward ECs by an intracrine signaling activation process. Here, we show that mECs in addition to microparticles release exerts a paracrine regulation to induce angiocyte formation.

Proangiogenic activity of TF-bearing mEMPs was unclear, especially in the context in which the signaling activity of TF seems to be linked to its cytoplasmic domain, which is probably sequestered within the microparticles. Some studies have demonstrated that cell surface TF signals through αβ-integrin and αββ1-integrin in ECs. Moreover, alternatively spliced TF also interact with αββ1-integrin and αββ1-integrin on ECs resulting in increase migration and tube formation, independently of FVIIa. Recently, it has been shown that alternatively spliced TF promotes breast cancer progression in a β1-integrin–dependent manner. In this regard, Collier and Ettelaie demonstrated that TF-bearing microparticles interact with cell surface β1-integrin in ECs to induce proliferation. Our findings further show that TF-bearing mEMPs interact with ECs through interaction with β1-integrin to stimulate angiocyte formation.

Integrins are transmembrane receptors that link proteins from outside the cell to cytoskeletal proteins and signaling pathways inside the cell. To investigate the signal transduction pathways involved in TF-bearing mEMPs, we studied the Rac1 protein. Rac1 is a small signaling G protein, member of the Rac subfamily of the RhGTPases family. In this study, we show that TF-bearing mEMPs induce mECs migration by binding β1-integrin and Rac1 and ERK1/2 activation. β1-integrin signals through Rac1, which plays a key role in connecting various cell surface receptors to biochemical pathways inducing cell motility and gene expression. Previous studies from our group demonstrated that endogenous TF in SMC promotes migration through Rac1, and Collier et al demonstrated that TF-bearing microparticles interact with ECs by interaction with β1-integrin to stimulate angiocyte formation.

That TF plays a role not only as a receptor but also as an effector. Normally, microparticles rich in TF derived from quiescent ECs have been linked to procoagulant activity. Moreover, TF-rich microparticles have been described in diseases associated with thrombotic complications, such as sepsis, diabetes mellitus, and particularly in cancer. TF-bearing microparticles may be released by tumor cells to increase new vessel formation. Our previous work showed that microvascular endothelial TF induces CCL2 expression and secretion that recruit SMC toward ECs by an intracrine signaling activation process. Here, we show that mECs in addition to microparticles release exerts a paracrine regulation to induce angiocyte formation.

Neovascularization can be considered as the culmination of several steps: first, degradation of extracellular matrix, migration, adhesion, and proliferation of ECs; second, formation of a new 3D tube, which then lengthens from its tip and circulation is re-established; and finally, recruitment of SMCs that must migrate as well, and adhere to the newly formed EC capillaries. The initial stimulus that leads SMCs to migrate is not well-understood. Our previous work showed that microvascular endothelial TF induces CCL2 expression and secretion that recruit SMC toward ECs by an intracrine signaling activation process. Here, we show that mECs in addition to microparticles release exerts a paracrine regulation to induce angiocyte formation.

Proangiogenic activity of TF-bearing mEMPs was unclear, especially in the context in which the signaling activity of TF seems to be linked to its cytoplasmic domain, which is probably sequestered within the microparticles. Some studies have demonstrated that cell surface TF signals through αβ-integrin and αββ1-integrin in ECs. Moreover, alternatively spliced TF also interact with αββ1-integrin and αββ1-integrin on ECs resulting in increase migration and tube formation, independently of FVIIa. Recently, it has been shown that alternatively spliced TF promotes breast cancer progression in a β1-integrin–dependent manner. In this regard, Collier and Ettelaie demonstrated that TF-bearing microparticles interact with cell surface β1-integrin in ECs to induce proliferation. Our findings further show that TF-bearing mEMPs interact with ECs through interaction with β1-integrin to stimulate angiocyte formation.

Integrins are transmembrane receptors that link proteins from outside the cell to cytoskeletal proteins and signaling pathways inside the cell. To investigate the signal transduction pathways involved in TF-bearing mEMPs, we studied the Rac1 protein. Rac1 is a small signaling G protein, member of the Rac subfamily of the RhGTPases family. In this study, we show that TF-bearing mEMPs induce mECs migration by binding β1-integrin and Rac1 and ERK1/2 activation. β1-integrin signals through Rac1, which plays a key role in connecting various cell surface receptors to biochemical pathways inducing cell motility and gene expression. Previous studies from our group demonstrated that endogenous TF in SMC promotes migration through Rac1, and Collier et al demonstrated that TF-bearing microparticles interact with ECs by interaction with β1-integrin to stimulate angiocyte formation.

Integrins are transmembrane receptors that link proteins from outside the cell to cytoskeletal proteins and signaling pathways inside the cell. To investigate the signal transduction pathways involved in TF-bearing mEMPs, we studied the Rac1 protein. Rac1 is a small signaling G protein, member of the Rac subfamily of the RhGTPases family. In this study, we show that TF-bearing mEMPs induce mECs migration by binding β1-integrin and Rac1 and ERK1/2 activation. β1-integrin signals through Rac1, which plays a key role in connecting various cell surface receptors to biochemical pathways inducing cell motility and gene expression. Previous studies from our group demonstrated that endogenous TF in SMC promotes migration through Rac1, and Collier et al demonstrated that TF-bearing microparticles interact with ECs by interaction with β1-integrin to stimulate angiocyte formation.
In vivo, TF-bearing mEMPs may be foes or friends. Procoagulant activity of microparticles has been reported in atherosclerotic plaques and in patients with acute coronary syndrome. They are considered as biomarkers of bad prognosis in cardiovascular disease; however, here we demonstrated that TF-bearing microparticles released by mECs programmed to form angioblasts, isolated and therapeutically administered to ischemic tissue are able to repair the consequences of arterial occlusion and promote postischemic revascularization. In conclusion, our data indicate that TF-bearing mEMPs can serve as a novel therapeutic intervention for the treatment of critical limb ischemia. TF-bearing mEMPs through β1-integrin signaling and finally CCL2 expression in skeletal muscles enhance reperfusion with increased blood flow and capillary formation around the ischemic zone. This capacity of TF to increase neovascularization leading to wound-healing and repair of ischemic injury by switching-on endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb Res. 2003;109:175–180.


overcome the consequences of arterial occlusion and tissue ischemia by promoting postischemic neovascularization and tissue reperfusion. These results indicate that TF-rich microvascular endothelial microparticles can operate in endothelial cells through β-integrin signaling. Delivery of TF-rich microvascular endothelial microparticles enhances collateral flow and capillary formation to induce reparative neovascularization in ischemic zones by releasing microparticles enriched in tissue factor (TF). We show that microvascular endothelial microparticles stimulates Akt/ERK-eNOS mediated angiogenesis and vascular maturation restoring blood flow in ischemic limbs of mice.

Significance

Therapeutic angiogenesis is a promising strategy for treating ischemia. Here, we describe that activated microvascular endothelial cells can induce reparative neovascularization in ischemic zones by releasing microparticles enriched in tissue factor (TF). We show that microvascular endothelial cells activation and phenotype change, from quiescent to proangiogenic, induce significant release of TF-rich microvascular endothelial microparticles. Delivery of TF-rich microvascular endothelial microparticles enhances collateral flow and capillary formation in the adductor muscles of mice in a hindlimb ischemia model. TF-rich microvascular endothelial microparticles increase angiogenesis operating in endothelial cells through β1-integrin signaling and CCL2 (chemokine [C-C motif] ligand 2) production that facilitates smooth muscle cell recruitment to form new and mature neovessels. These results indicate that TF-rich microvascular endothelial microparticles can overcome the consequences of arterial occlusion and tissue ischemia by promoting postischemic neovascularization and tissue reperfusion.
Angiogenic Microvascular Endothelial Cells Release Microparticles Rich in Tissue Factor That Promotes Postischemic Collateral Vessel Formation

Gemma Arderiu, Esther Peña and Lina Badimon

Arterioscler Thromb Vasc Biol. 2015;35:348-357; originally published online November 25, 2014;
doi: 10.1161/ATVBAHA.114.303927
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/2/348

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/01/09/ATVBAHA.114.303927.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIAL AND METHODS

Angiogenic microvascular endothelial cells release microparticles rich in tissue factor that promote post-ischemic collateral vessel formation

Gemma Arderiu PhD¹, Esther Peña PhD¹, Lina Badimon PhD¹,²
¹Cardiovascular Research Center (CSIC-ICCC). IIB-Sant Pau and Hospital de Sant Pau, Barcelona,
²Cardiovascular Research Chair Universitat Autònoma de Barcelona, Spain.
Materials and Methods

**Cell Culture**

Human dermal microvascular endothelial cells (HDMEC) (PromoCell) and immortalized human dermal microvascular endothelial cell line (HMEC-1)\(^1\) a kind gift from the Centre of Disease Control (Atlanta, GA), that behave similarly to HDMEC; were used. HDMEC and HMEC-1 were cultured in MCDB131 media (Invitrogen) and supplemented with 10% FBS, 2 mM glutamine (Invitrogen) and 50 mg/ml gentamicin (Invitrogen). These cells were used throughout the investigation because in contrast to other cells (tumor cells) they do not either express TF or release microparticles spontaneously. Both have the same characteristics, but HMEC-1 can be genetically manipulated, providing a unique model with which to generate TF overexpressing cells and release TF+EMPS.

Three-dimensional cultures were prepared on three-dimensional basement membrane (3DBM) (BD Matrigel™, BD Biosciences) as described.\(^2\) Cells were labeled with living cells fluorescent membrane dye, PKH26 Red Fluorescent Cell Linker Kit (Sigma).\(^3\) Cell movement was monitored by time lapse video microscopy at 15-minute intervals. Cells were viewed using a PL APO 20x/0.7 Multi-immersion CS. Images were acquired, digitalized, and processed with Leica Software TCS-AOBS.

**TF+**mEMPs- **Cell Culture Medium analysis**

Cell-derived microparticles were prepared by ultracentrifugation of cell supernatants that were harvested, clarified at 900 g for 15 minutes, and spun at 20,000 g for 45 minutes.\(^4,\)\(^5\) The microparticle pellet was resuspended and immediately used without freezing. Microparticle concentration was determined using the Zymuphen microparticle determination kit (Hyphen BioMed) and flow cytometry. Isolated EMPs were incubated with BD-horizon V450-conjugated annexin V (BD Biosciences) and a primary polyclonal anti-TF antibody (American Diagnostica) followed by goat anti-mouse Alexa 488 (Molecular Probes). Samples were diluted with Annexin V Binding Buffer (BD Biosciences) before being immediately analyzed on a FACSCantoll™ flow cytometer (BD Biosciences). TF bearing mEMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to anti-human TF mAb. The formation of MPs after cell activation is initiated by increase of intracellular calcium resulting in an elementary rearrangement of the phospholipid asymmetry with translocation of phosphatidyl-serine from the inner to the outer surface leaflet of the plasma membrane as a consequence of activation of scramblase and floppase/ABC1 and inhibition of translocase/flippase activities.\(^6,\)\(^7\) The presence of phosphatidylserine at the surface of the MPs membrane enables the use of the AnnexinV for MPs detection by flow cytometry. mEMP gate limits were established following two criteria: 1) calibration using a Flow-Check Size Range Calibration Kit (Polysciences)\(^8\) and 2) using an in vitro platelet-derived microparticle population as positive control. The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer. To identify TF positive marker events, thresholds were also set based on samples incubated with secondary antibody only. Data were analyzed with FACSDiva™ software (BD). The concentration (number of Annexin V and TF positive mEMP per μl of supernatants) was determined according to Nieuwland’s procedure, \(^9\) based on sample’s volume, flow cytometer’s flow rate and the number of fluorescence- positive events.

Background cell auto fluorescence was assessed by omission of the primary antibody. Total TF antigen and activity levels were measured using an IMUBIND® TF ELISA Kit (Sekisui Diagnostica).

**Generation of TF bearing mEMPs: TF overexpressing cells and microparticle isolation**

To enrich the number of TF bearing mEMPs (TF+mEMPs), we performed TF overexpressing cells. The generation of lentiviruses expressing TF-GFP has previously been described in detail in Arderiu et al.\(^10\) mECs were infected with pLVx-TF-AcGFP1 encoding for a full length TF. 1x10^7 TF-GFP overexpressing cells were induced to migrate by injuring with multiples scratches (more than 60% of wounded area) for 48 hours to stimulate microparticles
release.\textsuperscript{11} Cell-delivered microparticles from TF-GFP overexpressing HMEC-1 cells were prepared as described above. The microparticle pellet was resuspended and immediately used without freezing. To ensure consistency between the TF+mEMPs preparations, microparticles-TF concentration, TF antigen and levels of TF activity of the microparticles isolated from nontransfected or TF-GFP-transfected mECs were determined before use (Supplementary Table I). MP-TF concentration was measured by Zymuphen MT-TF determination kit, the concentration was established respectively to an internal standard and it was expressed as TF antigen equivalents (pM). Experiments were performed with 100 pmol/L of TF+mEMPs, into the range of TF concentrations corresponding to those found in plasma of cardiovascular disease patients (50 to 200 pmol/L).\textsuperscript{12}

**Generation of TF poor mEMPs: Silencing RNA**

Delivery of small interfering RNAs (siRNAs) into mECs was done with a Nucleofector device and its corresponding kits (Amaxa, Inc.). Transfection protocols were performed following manufacturer’s instructions using the T16 and A33 programs respectively. The scrambled siRNA or annealed pre-designed siRNA against TF (s4932) or PAR2 (s4926) were purchased from Applied Biosystems. After transfection, cells were plated into matrigel and 18 hours later RNA and protein were isolated. In all experiments, a scrambled siRNA was used as control to evidence unspecific changes in gene expression profile or in cell phenotype that may have resulted from the use of siRNA. TF silenced cells were forced to migrate for 48 hours to stimulate microparticles release. TF poor mEPs (TF-mEMPs) were obtained as described above.

**ECs adhesion assay**

TF+mEMPs were used to coat 96-well tissue culture plates; collagen served as positive control. 2 x 10\textsuperscript{4} mECs/well were added to 96-well plates and left to adhere (5% CO\textsubscript{2} at 37°C for 2 hrs). Following the incubation, non-adherent cells were removed by washing the wells twice with PBS. The adherent cells were fixed and stained with Diff-Quick (VWR Scientific Products) and counted at x10 using phase-contrast inverted microscope. To study integrin interaction cells were incubated with integrin-blocking antibodies. Integrin β1 function blocking monoclonal antibody (clone P5D2) was obtained from Millipore Corporation.

**CCL2/MCP-1 assay**

Quantitative determination of CCL2 concentrations in cell culture supernatant was determined using a Quantikine® Human CCL2/MCP-1 immunoassay (R&D Systems).

**Western blot analysis**

Total protein was isolated from lysates of mECs after 18 hours cultures on 3D Matrigel or from tissue obtained from the euthanatized animals. Equal amounts of reduced protein were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking for nonspecific binding, western-blots were probed with a monoclonal anti-TF antibody (1:1000) (American Diagnostica), polyclonal anti-Rac1 antibody (1:1000), polyclonal anti-p44/42 MAPK (137F5) antibody (1:1000) and polyclonal anti-phospho p44/42 MAPK (Thr202/Tyr204) antibody (1:1000) (Cell Signaling), and monoclonal anti-PAR2 antibody (1:1000) (Santa Cruz Biotechnology), followed by goat anti-mouse-HRP (1:2000) or donkey anti-rabbit-HRP (1:5000). Excess of antibody was removed by extensive washing and blots were developed by ECL system (Amersham Biosciences). The membranes were then stripped and treated with polyclonal anti-β-actin antibody (1:1000) (Abcam Inc. Cambridge, MA), followed by donkey anti-rabbit-HRP (1:5000) and detected by ECL system. Band densities were determined with the ChemiDoc™ XRS system (Bio-Rad) in chemiluminescence detection modus and Quantity-One software (Bio-Rad).
**Immunofluorescence staining**
Cells were cultured in 3DBM for 18 hours, fixed in 3.5% paraformaldehyde, washed with 50 mmol/L PBS/glycine thrice for 20 minutes at room temperature, blocked with 10% goat serum in immunofluorescence buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20 in PBS) for 1 hour at room temperature, and with 2.5% BSA with 1:100 Fab fragment (anti-mouse IgG, Fab fragment of goat antibody, Sigma) in immunofluorescence buffer for 45 minutes at room temperature. Incubation with the primary antibody anti-TF was done at 1:50 dilution in immunofluorescence buffer plus 1:100 fab fragment for 1 hour at room temperature. After washing with immunofluorescence buffer, 1:100 Alexa Fluor anti-rabbit 488 IgG (Molecular Probes) was applied for 1 hour at room temperature. Nuclei were counterstained with Hoechst 33342. Controls were stained with secondary antibodies only. Stained cells were washed and covered with Prolong Gold antifade reagent (Molecular Probes). Images of immunostained cells were recorded on a Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany). Cells were viewed with HCX PL APO 63x/1.2 W Corr/0.17 CS objective. Fluorescent images were acquired in a scan format of 1024 x 1024 pixels in a spatial data set (xyz or xzy) and were processed with the Leica Standard Software TCS-AOBS.

**Animals**
We used nude male mice of 6-7 week old (R/SOPF BALB/C NU/NU CBy.Cg-Foxn1 nu/j, Charles River Laboratories, France). Animal care and experimental procedures were approved and followed by the Ethics Committee of Cardiovascular Research Center.

**Mouse aortic ring model**
Mouse thoracic aortas were isolated and cleaned of the surrounding tissue in serum-free RPMI (Invitrogen) containing 50 mg/ml gentamicin. Dissected aortas were flushed, cut into equal segments, embedded in matrigel, and covered with MCDB 131 containing 10% serum and gentamicin. Sprouts were counted on day 4.

**Angiogenesis assay in vivo**
300 µl of Matrigel media was mixed with PBS, VEGF/bFGF (50ng/ml each), TF*mEMPS (100 pM) and recombinant TF protein (100 pM) (ProSpec-Tany TechnoGene Ltd. Israel) were injected subcutaneously in nude mice. After injection, the Matrigel rapidly formed a plug. All mice were euthanized at seven days post-injection and the skin of the mouse was easily pulled back to expose the matrigel plug, which remained intact. Underlying skins of plugs were visualized macroscopically (Leica AF 6000LX Stereo Microscope with Digital camera DFC, 8 bits of Resolution, Objective 1.0x0.03/1.0x0.09) or were fixed, embedded, and stained with appropriate antibodies.

**Unilateral hind limb ischemia model in mice**
Male mice at the age of 6-7 weeks were used. The mice were anesthetized with 1.5% isoflurane in air. Body temperature was closely monitored and maintained between 32 and 35°C. After incision of the skin in the inguinal region, the femoral artery was separated from the femoral vein and nerve. Both common iliac artery and femoral artery in the left side were tightly ligated with 6/0 Ethilon sutures. Subsequently, the skin incision was closed by sutures. The arteries at the right side were not ligated and served as control. A total of 36 nude mice were divided into 6 groups: 1) sham, in which the left limb was opened without femoral artery ligation (sham group); 2) the left femoral artery was ligated to induce hind limb ischemia and 24 hours post surgery 100 µl of PBS were injected intramuscularly (HLI + PBS); 3) same as for the previous group (2), but with 100 pM recombinant TF protein injected intramuscularly (HLI + TF); 4) same as for the PBS group (2), but with intramuscular injection of 100 pM TF*mEMPS (HLI + TF*mEMPS); 5) same as for the second group, but with injection of 1x10^7 TF overexpressing HMEC-1 cells (HLI + ECsTF+); and 6) same as for the second group, but with injection of 1x10^7 TF silenced HMEC-1 cells (HLI + ECsTF-). All...
these procedures were carried out 24 h after surgery and the injections were made into 4 delivery sites in the ischemic area (25 µl for each site).

The blood flow of ischemic (left) and contra-lateral non-ischemic (right) hind limbs was measured with Vevo® 2100 System (VisualSonics Inc.) before and after surgery (Supplementary Figure I). After 21 days all mice were euthanized and the thigh muscle was excised. For immunohistochemistry, whole ischemic and nonischemic limbs were immediately fixed, embedded in O.C.T. and stained with appropriate antibodies for histological examination. For total protein extraction, isolated tissue samples were rinsed, snap-frozen in liquid nitrogen, and stored at -80°C until use.

**Assessment of limb necrosis and active hind limb movement.**

Tissue necrosis was scored as described in Qi et al. 2010:13 “0” was for no necrosis, “1” for necrosis of one toe, “2” for necrosis of two or more toes “3” for necrosis of the foot, “4” for necrosis of the leg, and “5” for auto-amputation of the entire leg. Moreover, the severity of ambulatory impairment was assessed by using the following scale as described in Qi et al. 2010: “0” was for normal response (plantar/toe flexion in response to tail traction), “1” for plantar but not toe flexion, “2” for no plantar or toe flexion, “3” for dragging of foot, and “4” for spontaneous movement of non-ischemic hind limb. Physical examinations of both tissue necrosis and ambulatory impairment were performed by an observer who was blinded to treatments, but all animals with paralysis or necrosis of the hind limb/ toes were euthanized according to the protocol for assessing animal welfare and they were excluded from the study.

**Magnetic resonance angiography**

After 21 days post-surgery, animals were anesthetized and maintained on 1.5% isoflurane in air and body temperature was closely monitored and maintained between 32 and 35°C using warm air during all imaging acquisition. Magnetic resonance angiography was used to evaluate the spontaneous vascular adaptation to HLI or subsequently to application of TF-mfEMPS in the muscle.14

In *vivo* 1H-magnetic resonance angiography (MRA) studies were performed at the NMR facility (SeRMN) of the Autonomous University of Barcelona in a 7T Bruker BioSpec 70/30 USR (Bruker BioSpin GmbH, Ettlingen, Germany) system equipped with a mini-imaging gradient set (400mT/m) and using a quadrature transceiver volume coil (35 mm inner diameter). MR data were acquired and processed on a Linux computer using Paravision 5.1 software (Bruker BioSpin GmbH, Ettlingen, Germany). Mice were positioned in a custom-built bed, which allowed delivery of anesthesia (isoflurane, 1.5-2.0% in O2 at 1 L/min). Respiratory frequency was monitored with a pressure probe and kept between 60–80 breaths/min. Body temperature was measured with a rectal probe and controlled using a warm air heating system. T2-weighted fast spin-echo images were initially obtained in axial and coronal planes to be used as reference scout images. Imaging parameters for these images were: effective echo time (TEeff)=36 ms, repetition time (TR)=3 s, echo train length (ETL)=8, field of view (FOV)=4x4cm², matrix size (MTX)=128x128, slice thickness (ST)=1.5mm. MRA was acquired using a multi-slice 2D time of flight flow compensated sequence in axial planes with TE=4.5ms, TR=18ms, flip angle=80º, FOV=3x3cm², and MTX= 256x256, collecting 120 slices with a pack extend of 3 cm (ST=0.4mm and interslice distance=0.25mm). MRA data sets were interpolated in the slice direction to 256, and the resulting 3D 256³ data were visualized using maximum intensity projection (MIP) reconstruction. Total acquisition time was 20 minutes.

**Histological assessment of neovascularization: Analysis of capillary density**

Blood vessels were visualized by immunofluorescence staining. Five-micrometer-thick frozen skin sections or adductor muscle were prepared for immunostaining. We used rabbit anti-Von Willebrand factor antibody (1:50) (Abbiotec), this was followed by incubation with Alexa 488-conjugated goat anti-rabbit antibody (1:100) (Molecular Probes) or monoclonal anti-α-smooth muscle actin FITC conjugated (1:200) (Abcam). Cell nuclei were
counterstained with Hoechst 33342 (1:1000) (Molecular Probes). Stained cells were washed and covered with Prolong Gold antifade reagent (Molecular Probes). Images of immunostained cells were recorded on a Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany). Cells were viewed with HCX PL APO 20x/0.7 Multi-immersion CS objective. Fluorescent images were acquired in a scan format of 1024 x 1024 pixels in a spatial data set (xyz or xzy) and were processed with the Leica Standard Software TCS-AOBS. Controls with no primary antibody showed no fluorescence labeling.

**Thromboelastographic coagulation analysis**
The extrinsic and intrinsic clotting time in plasma of HLI animals was measured. Dynamic whole blood clot formation was performed with the ROTEM coagulation analyzer (Pentapharm, Germany), which is based on the thrombelastograph system using the extrinsically activated Ex-TEM assay, and the intrinsically activated In-TEM assay.

**Statistical analysis**
For comparisons between two groups, statistical analyses were performed using the two-sample independent-groups $t$ test. Comparison of multiple mean values was made by one-way ANOVA, and statistical significance among multiple groups was determined by Tukey's post-hoc test (for pair-wise comparisons of means). All results are presented as mean ± SEM from at least three independent experiments. $P < 0.05$ was considered statistically significant.
References


Angiogenic microvascular endothelial cells release microparticles rich in tissue factor that promote post-ischemic collateral vessel formation

Gemma Arderiu PhD, Esther Peña PhD, Lina Badimon PhD

1 Cardiovascular Research Center (CSIC-ICCC). IIB-Sant Pau and Hospital de Sant Pau, Barcelona,
2 Cardiovascular Research Chair Universitat Autònoma de Barcelona, Spain.
Supplemental Table 1

<table>
<thead>
<tr>
<th>Source of microparticle s</th>
<th>mEMPs AnnV+ x10^6/ml</th>
<th>mEMPs AnnV+TF+ x10^6/ml</th>
<th>TF antigen concentration (ng/ml)</th>
<th>TF activity (U/ml)</th>
<th>TF concentration (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDMEC</td>
<td>2.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>0.1 ± 0.002</td>
<td>2.5 ± 1.2</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>2.7 ± 0.2</td>
<td>1.8 ± 0.4</td>
<td>0.133 ± 0.004</td>
<td>2.9 ± 0.8</td>
<td>4.5±0.5</td>
</tr>
<tr>
<td>TF-silenced HMEC-1</td>
<td>2.6 ± 0.35</td>
<td>0.2 ± 0.05</td>
<td>0.051 ± 0.005</td>
<td>0.05 ± 0.002</td>
<td>2±0.75</td>
</tr>
<tr>
<td>TF-overexpressing HMEC-1</td>
<td>3.4 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>4.98 ± 0.04</td>
<td>44.38 ± 6.3</td>
<td>100±10</td>
</tr>
</tbody>
</table>

Table I: Characteristics of microvascular endothelial microparticles: mEMPs/ml corresponding to total number of events for annexin V+ (A) and double positive for annexin V and TF (B); TF antigen concentration (C), activity (D) and TF concentration of endothelial microparticles. Microparticles from HDMEC or control HMEC-1, TF silenced cells or TF overexpressing cells. 1x10^7 cells were induced to migrate for 48 hours to stimulate microparticles release. EMPs/ml was analyzed by flow cytometry (A and B), TF antigen was measured using an IMUBIND® TF ELISA Kit (Sekisui Diagnostica) (C), activity was measured as described in Peña et al. (D) and TF concentration was measured by Zymuphen MT-TF determination kit (E).
Supplemental Figure I

**Micro-ultrasound of the legs before and after surgery**
The blood flow immediately before and after surgery was measured using a Vevo 2100 High-Resolution Microultrasound System (Visualsonics Inc, Amsterdam, Netherlands) The Microultrasound System allows for repeated, noninvasive and quantitative measurements of the blood flow of the certain sites of the artery. The mice were anesthetized with 1% isoflurane, placed on a heat pad, and kept at 37°C to minimize data variations caused by fluctuating body temperatures.
Supplemental Figure II

(A) Flow cytometry analysis of supernatants from HMEC-1, bar graphs shows quantification of mEMPs in medium of quiescent cells or cells forming tube like structures (angiogenic cells). Results are expressed as mEMPs/ml corresponding at total number of events, positive annexin V or double positive for annexin V and TF, data are shown as mean ±SEM of 3 independent experiments. Statistical analysis was performed by Student’s t-test, ***P<0.001 vs quiescent cells.

(B) Flow cytometry analysis of supernatants from HMEC-1, bar graphs shows quantification of mEMPs in medium of migrating cells, control cells (WT) or TF overexpressing cells (TF+). Results are expressed as mEMPs/ml corresponding at total number of events, positive annexin V or double positive for annexin V and TF, data are shown as mean ±SEM of 3 independent experiments. Statistical analysis was performed by Student's t-test (**P<0.001).

(C) Flow cytometry analysis of supernatants from HMEC-1 cultured on matrigel for 18 hours, bar graphs shows quantification of EMPs in medium of control cells (WT) or TF siRNA transfected cells (TF-). Results are expressed as mEMPs/ml corresponding to the number of double positive events for annexin V and TF; data are shown as mean ±SEM of 3 independent experiments. Statistical analysis was performed by Student's t-test (**P<0.001).
Supplemental Figure III

**A**

Real-time polymerase chain reaction analysis of TF mRNA levels in mECs with TF+EMPs in presence or absence of β1-integrin blocking antibody.

**B**

Western blots show the TF expression found in mECs treated with TF+mEMPs, but β1-integrin blocking antibody inhibits TF transfer from TF+mEMPs to mECs (n=3).

**TF+EMPs transfer TF to endothelial cells. (A)** Real-time polymerase chain reaction analysis of TF mRNA levels in mECs with TF+EMPs in presence or absence of β1-integrin blocking antibody. **(B)** Western blots show the TF expression found in mECs treated with TF+mEMPs, but β1-integrin blocking antibody inhibits TF transfer from TF+mEMPs to mECs (n=3).
Supplemental Figure IV

Real time polymerase chain reaction analysis of $\beta$1-integrin mRNA levels in scrambled or $\beta$1-integrin mECs in presence or absence of TF$mEMPs$
Supplemental Figure V

- + + TF\textsuperscript{+}mEMPs
- - + Hirudin

TF
ETS1
CCL2
\(\beta\)-actin

**CLL2 expression is independent of thrombin formation.** Western blots show TF, ETS1 and CCL2 expression in mECs in presence of TF\textsuperscript{+}mEMPs with and without 50 nM of hirudin. To test the equal loading, the western blots were reproved for \(\beta\)-actin.
TF+EMPs activated β1-integrin/Rac1/ERK1/2 pathway is independent of PAR2. Western blots show PAR2 and TF expression in mECs in presence of TF+mEMPs: cells transfected with scrambled (Sc) siRNA and cells transfected with PAR2-siRNA as indicated. To test the equal loading, the western blots were reproved for β-actin. Histogram shows densitometric analysis of the average levels for total protein to β-actin, results are expressed as ±SEM. ***P<0.001.
Supplemental Figure VII

Western Blot analysis of extracts from TF overexpressing HMEC-1 cells and recombinant TF protein, using mouse anti-TF, goat anti-TF or rabbit anti-TF antibody.
Supplemental Figure VIII

**A.** Western Blot showing VEGFR protein levels or VEGF %mRNA levels in HMEC-1 cells in presence or absence of TF+mEMPs (100 pmol/L) cultured in 3DBM for 18 hours (n=3).

**B.** Western blot show VEGFR protein levels in skin tissue surrounding matrigel plugs containing PBS (control), VEGF/bFGF (50ng/ml each) or TF+mEMPs (100 pmol/L). (n=3).
Supplemental Figure IX

Western blots showing full length TF (flTF) and soluble TF (sTF) proteins levels in skin tissue surrounding matrigel plugs containing: control solvent (PBS); VEGF/bFGF (50 ng/ml each); TF*mEMPs (100 pM); or recombinant TF (100 pM). To test the equal loading, the western blots were reproved for β-actin.
**Supplemental Figure X**

**A.** Upper images show a macroscopic view of representative skin tissue 7 days after injection of matrigel plugs, and low panels show immunofluorescence staining for von Willebrand factor positive cells. Matrigel plugs contain: control solvent (PBS); TF+mEMPs (100 pM); or TF+mEMPS (100pM) with hirudin (10mg/Kg). **B.** Quantification of tube % covered area. Values represent means ± SD from 4 animals for each group. Statistical analysis was performed by Student’s t-test (**p<0.001 vs control cells). **C.** Western blots show TF, ETS1, CCL2, and β-actin.

**TF*mEMPs induce angiogenesis in vivo independently of thrombin formation.** **A.** Upper images show a macroscopic view of representative skin tissue 7 days after injection of matrigel plugs, and low panels show immunofluorescence staining for von Willebrand factor positive cells. Matrigel plugs contain: control solvent (PBS); TF*mEMPs (100 pM); or TF*mEMPS (100pM) with hirudin (10mg/Kg). **B.** Quantification of tube % covered area. Values represent means ± SD from 4 animals for each group. Statistical analysis was performed by Student’s t-test (**p<0.001 vs control cells). **C.** Western blots show TF, ETS1, CCL2, and β-actin.
and CCL2 protein levels in skin tissue. To test the equal loading, the western blots were reproved for β-actin. Aortic segments from nude mice were implanted into matrigel supplemented with: solvent control (PBS); TF+mEMPs (100 pM); TF+mEMPS (100pM) with hirudin (500 nM). Outgrowing sprouts were visualized and counted on day 4. Images are representative from four independent experiments.