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Abstract

Background. Tissue factor (TF) is an important trigger of arterial thrombosis. The green tea catechin epigallocatechin-3-gallate (EGCG) is a ligand of the 67 kDa laminin receptor (67LR) and exhibits cardioprotective effects. This study investigates whether 67LR regulates TF expression in human endothelial cells.

Methods and Results. Immunofluorescence demonstrated that human aortic endothelial cells expressed 67LR. Cells grown on laminin expressed 35% less TF in response to TNF-alpha (TNF-α) than those grown on fibronectin (n=6; p<0.001). EGCG (1-30 µM) inhibited TNF-α and histamine induced endothelial TF expression and activity in a concentration dependent manner resulting in 87% reduction of TF expression (n=5; p<0.001); in contrast, expression of tissue factor pathway inhibitor was not affected (n=4; p=NS). In vivo administration of EGCG (30 mg/kg/d) inhibited TF activity in carotid arteries of C57BL6 mice. Real-time PCR and promoter studies revealed that EGCG decreased TF expression at the transcriptional level and impaired activation of the mitogen activated protein (MAP) kinase JNK 1/2, but not ERK or p38. Similarly, the JNK 1/2 inhibitor SP600125 (1 µM) impaired TF promoter activity (n=4; p<0.001) and protein expression (n=4; p<0.001). 67LR blocking antibodies blunted the inhibitory effect of EGCG on both TF protein expression and JNK activation. In contrast, vascular cell adhesion molecule 1 (VCAM-1) was not affected by laminin nor EGCG, and its expression was not regulated by JNK. EGCG did not affect TNF-α stimulated NFκB activation.
**Conclusions.** Laminin receptor activation inhibits endothelial TF expression by impairing JNK phosphorylation. Thus, 67LR may be a potential target for the development of novel anti-thrombotic therapies.

**Key Words:**

Laminin; 67-kDA Laminin Receptor; Tissue Factor; MAP Kinase, Epigallocatechin-3-Gallate
Introduction

Tissue factor (TF) plays an important role in initiating coagulation [1]. Inflammatory mediators such as TNF-alpha (TNF-α) or histamine are potent inducers of TF expression in vascular cells [2]. Elevated levels of TF are detected in atherosclerotic plaques, and an involvement of TF in drug-eluting stent thrombosis has been discussed as well [3,4]. These findings suggest that modulation of TF expression in vascular cells may offer a strategy for the treatment and prevention of arterial thrombosis.

The extracellular matrix (ECM) plays a pivotal role in vascular homeostasis, since it maintains vascular integrity by regulating proliferation, migration, and morphogenesis of endothelial cells [5,6]. Indeed, the basement membrane forms a scaffold around the endothelial tube, and interactions between endothelial cells and constituents of the basement membrane are essential for vessel wall integrity [7]. The non-collagenous glycoprotein laminin is a cross shaped heterotrimer of α, β, and γ subunits and a major component of the basement membrane [7]. The membrane bound 67 kDa-laminin receptor (67LR) is a non-integrin cell surface receptor directly interacting with the β1 subunit of laminin [8]. Besides its role in cell adherence to the extracellular matrix, there is growing evidence that 67LR activation induces functional changes within cells [8]. 67LR has indeed been implicated in shear stress induced endothelial nitric oxide synthase (eNOS) activation, since its blockade abolished shear induced eNOS phosphorylation [9]. Despite such observations,
the role of 67LR in endothelial activation remains incompletely understood, and possible implications for arterial thrombosis have not been investigated.

Recently, 67LR has been identified as a cell surface receptor for the green tea polyphenol epigallocatechin-3-gallate (EGCG) [10]. EGCG is the most abundant polyphenol found in green tea and constitutes 30% of its dry mass. Experimental studies revealed cardioprotective properties of ECGC consisting in anti-inflammatory, anti-oxidant, and anti-atherogenic effects [11-16]; moreover, large epidemiological trials demonstrated that green tea consumption is inversely associated with cardiovascular mortality [17-19].

The present study investigates whether 67LR modulates TF expression in human aortic endothelial cells.
Methods

Cell culture

Human aortic endothelial cells (HAEC; Clonetics, Allschwil, Switzerland), human umbilical venous endothelial cells (HUVEC; Clonetics) and human aortic vascular smooth muscle cells (HASMC, Clonetics) were cultured on fibronectin as described [20]. Cells were rendered quiescent in medium supplemented with 0.5% fetal bovine serum for 24 hours before stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) or 10 μM histamine (Sigma, St. Louis, MO). Cells were pretreated with EGCG (Sigma) for 60 minutes. For studies of VCAM-1 and TF protein expression HAEC were stimulated for 5 hours. To block the mitogen activated protein (MAP) kinase c-Jun terminal NH₂ kinase 1/2 (JNK 1/2), HAEC were treated with SP600125 (Calbiochem, Lucerne, Switzerland) for 60 minutes before stimulation. Cytotoxicity was assessed using a colorimetric assay for detection of lactate dehydrogenase release (Roche, Basel, Switzerland). Where mentioned, dishes were coated with laminin (25 μg/mL; Sigma). To compensate for the slower growth rates of HAEC on laminin as compared to fibronectin, a higher cell number was seeded on the dishes; the two groups exhibited similar confluency at the time of the experiments. To study the role of 67LR, cells were pretreated for 60 minutes with either a monoclonal (MLuC5, 1:1’000) or a polyclonal (ab711, 1:1’000) anti-human 67LR blocking antibody or the appropriate isotype antibodies (all from Abcam, Cambridge, UK).
**Immunofluorescence microscopy**

HAEC were grown on LabTek chamber slides (Nunc, Rochester, MN), washed three times with PBS, and fixed with 4% paraformaldehyde for 15 min. Cells were washed twice with PBS and incubated with anti-67LR (MLuC5) antibody for 1 hour (1:1’000), followed by incubation with Alexa-488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 1 hour (1:2’000). Antibodies were diluted in PBS containing 1% bovine serum albumin. After washing with PBS, cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA), mounted on chamber slides, and examined by a fluorescence microscope (Olympus, DP40, Tokyo; Japan).

**Western blot**

Protein expression was determined by Western blot analysis. Antibodies against human TF, tissue factor pathway inhibitor (TFPI) (both from American Diagnostica, Stamford, CT), and vascular cell adhesion molecule-1 (VCAM-1; R&D Systems, Minneapolis, MN) were used at 1:2500 dilution. Antibodies against phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (extracellular signal regulated kinase [ERK]), and c-Jun NH2 terminal kinase (JNK1/2; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK1/2 (all from Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. IkB-α expression pattern was assessed by anti-human IkB-α antibody (1:5000; Cell Signaling). All blots were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:10’000...
dilution, Chemicon International, Temecula, CA). The quantification of protein was performed by densitometric analysis using Scion Image Software (Scion Corp., Frederick, MD).

**TF surface activity**

TF surface activity was analyzed on HAEC after 5 hours of stimulation with TNF-α in the presence or absence of EGCG (30 μM) using a colorimetric assay (American Diagnostica). Briefly, TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. The absorbance of the reaction mixture was measured at 405 nm, and TF activity was determined from a standard curve performed with lipidated human TF.

**In vivo study**

Analysis of TF activity was performed in 10 week old male C57BL6 mice (Jackson Laboratories, Bar Harbor, ME) weighing an average of 26 g and fed a normal chow diet (KLIBA NAFAG, Kaiseraugst, Switzerland). EGCG was dissolved in PBS (pH 7.4) and administrated i.p. at a dose of 30 mg/kg/d for 7 days. Age, sex, and weight matched controls received an equal volume of vehicle. Mice were then euthanized and the left common carotid artery harvested for analysis of TF activity using a colorimetric assay (American Diagnostica).

**Real-time PCR**
RNA was extracted from HAEC using TRIzol Reagent (Invitrogen, Carlsbad, CA) and real-time PCR performed as described [20]. The following primers were used: for full length human TF (F3): sense 5’-TCCCCAGAGTCACACCTTACC–3’ (bases 508-529 of F3 cDNA; NCBI no. NM 001993), antisense 5’–CCTTTCTCCTGGCCCATACAC–3’(bases 843-863 of F3 cDNA; NCBI no. NM 001993); for human ribosomal L28: sense 5’-GCATCTGCAATGGATGGT-3’, antisense 5’–CCTTTCTCCTG-GCCCATACAC-3’. Melting curve analysis was performed after amplification to verify the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for TF and L28, a standard curve generated from serial dilutions of purified amplicons was included.

**TF promoter activity**

TF promoter activity was measured as described [21]. Briefly, the minimal TF promoter (-227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene using a pGL2-Basic plasmid (Promega, Madison, WI). For transduction, the adenoviral vector Ad5/hTF/Luc was added to HAEC at an multiplicity of infection of 100 plaque forming units/cell for 1 hour and then removed. The adenoviral vector VQAd Empty was used as a negative control. HAEC were kept in growth medium for 24 hours and then serum starved for 24 hours prior to TNF-α stimulation for 1 hour with or without EGCG (30 µM). Firefly luciferase activity was determined in cell lysates using a luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized by determining
protein concentration in the lysates.

**Transfection with siRNA**

HAEC were transfected with siRNA (15 nM) against JNK 1 (5'-AAAGAAUGUCCUACCUCUTT-3'), JNK 2 (5'-AGAAGGUAGGACAUUCUUUTT-3') or scrambled RNA (all from Sigma) using the N-TER™ Nanoparticle siRNA Transfection System (Sigma) for 4 hours. After transfection, HAEC were grown for 12 hours and then serum starved for 24 hours prior to the experiment.

**Nuclear extraction and NFkB activity assay**

For NFκB activity measurements, HAEC were stimulated with TNF-α for 30 minutes in the presence or absence of EGCG (30 μM). Nuclear extracts were obtained from HAEC using a nuclear extraction kit (Active Motif, Carlsbad, USA). Nuclear protein (20 µg) was loaded in each well, and NFkB activity was measured using a TransAM NFkB p65 kit (Active Motif) according to manufacturer’s recommendations.

**Statistics**

Data are presented as mean±SEM. Statistical analysis was performed using two tailed unpaired Student’s t-test or one-way ANOVA as appropriate. A value of p<0.05 was considered significant. All results are representative of at least four independent experiments.
Results

Laminin inhibits TF protein expression

Immunofluorescence microscopy revealed that 67LR was abundantly expressed on the surface of HAEC (Figure 1A). Western blot analysis demonstrated that TNF-α (5ng/mL) induced TF expression was 35% lower in cells growing on laminin as compared to those growing on fibronectin (n=4; \( p<0.001 \); Figure 1B).

67LR inhibits TF protein expression

EGCG (1-30 µM) inhibited TNF-α (5 ng/mL) induced TF expression in a concentration dependent manner (n=5; *\( p<0.001 \); figure 2A); the maximal effect occurred at 30 µM and decreased TF expression by 87% as compared to TNF-α alone. Similarly, EGCG (30 µM) inhibited histamine (10 µM) induced TF expression by 84% as compared to histamine alone (n=5; *\( p<0.001 \); figure 2B). EGCG did not affect expression of TFPI (n=4; \( p=\text{NS} \); supplemental figure 1). The effect of EGCG on TF protein expression was paralleled by a reduced TF surface activity (n=4; *\( p<0.001 \); figure 2C). Further the effect of EGCG on TF activity was assessed in carotid artery lysates of C57BL6 mice. EGCG administrated for 7 days (30 mg/kg/d) reduced TF activity by 46.6±17% as compared to control animals (n=5; \( p<0.05 \); figure 2D). In line with this observation, EGCG also impaired TNF-α induced TF expression in cultured HASMC and HUVEC (n=4; \( p<0.01 \); supplemental figure 2 and 3). Pretreatment of HAEC with the monoclonal 67LR blocking antibody MLuC5 blunted the
inhibitory effect of EGCG on TF protein expression as compared to cells treated with the isotype control (n=4; *p<0.001 vs TNF-α alone; figure 2E). A similar effect was observed using a polyclonal 67LR antibody (n=4; *p<0.001; data not shown). LDH release was not altered by any of the EGCG concentrations used (n=4; p=NS; data not shown).

67LR inhibits TF mRNA expression and promoter activity but not TNF-α induced NFκB activation

TNF-α induced TF mRNA expression within 2 hours of stimulation as determined by real-time PCR analysis (n=5; *p<0.0001; Figure 3A). EGCG (30 µM) impaired TNF-α induced TF mRNA expression by 74% as compared to TNF-α alone (n=5; *p<0.001; Figure 3A). EGCG (30 µM) did not alter TNF-α stimulated IκB-α degradation nor TNF-α induced nuclear translocation and DNA binding activity of the NF kappa B subunit p65 (n=4; p=NS, figure 3B and 3C). To assess whether the effect on TF mRNA was mediated via promoter inhibition, the impact of EGCG on TF promoter activity was analyzed after adenoviral transduction of endothelial cells with firefly luciferase under control of the human minimal TF promoter (-221 bp to +121 bp). TNF-α enhanced TF promoter activity by 2.2 fold as compared to control conditions (n=4; *p<0.001; Figure 3D); while EGCG (30 µM) inhibited this effect by 51% (n=4; *p<0.001; Figure 3D).

67LR inhibits JNK1/2 phosphorylation

To determine whether 67LR inhibited MAP kinases, phosphorylation of
these mediators was assessed at different time points after TNF-α stimulation. TNF-α (5 ng/mL) transiently activated JNK1/2, ERK, and p38; maximal activation was observed after 15 minutes and returned to basal levels within 60 minutes (Figure 4A). EGCG inhibited phosphorylation of JNK by 46% as compared to TNF-α alone (n=4; *p<0.01; Figure 4B), while phosphorylation of ERK and p38 was not altered (n=4; p=NS; Figure 4B). Total expression of JNK 1/2, ERK, and p38 remained unaffected at all time points examined (Figure 4A). Targeting of JNK1 and JNK2 with specific siRNA impaired TNF-α induced TF expression by 33% and 60%, respectively as compared to TNF-α alone (n=4; p<0.05 for JNK1 vs TNF-α alone and p<0.01 for JNK2 vs TNF-α alone; figure 5A). Inhibition of JNK 1 and 2 phosphorylation with SP600125 mimicked the effect of siRNA on TF protein expression (n=4; *p<0.001; Figure 5B) and abolished TNF-α induced TF promoter activation (n=4; *p<0.001; Figure 5C). The inhibitory effect of EGCG on JNK phosphorylation was prevented by pretreatment with the 67LR blocking antibody MLuC5 (n=3; p<0.01; Figure 5D).

**Neither 67LR nor JNK 1/2 regulate VCAM-1 expression**

Western blot analysis revealed that TNF-α induced VCAM-1 expression (n=4; p<0.001; Figure 5A) in HAEC. Neither 67LR activation by laminin or EGCG (1-30 μM) nor JNK inhibition by SP600125 (n=4; p=NS; Figure 6A, 6B, and 6C) impaired TNF-α induced VCAM-1 expression. Similar to the result obtained in HAEC with EGCG in HUVEC (n=4; p=NS; supplemental figure 4).
Discussion

This study demonstrates that activation of the 67LR by laminin or EGCG inhibits TNF-α induced TF expression in endothelial cells. Moreover, administration of EGCG in C57BL6 mice diminishes TF activity in carotid artery lysates. This effect is mediated at the transcriptional level via reduced phosphorylation of the JNK isoforms p46 and p54 (JNK1 and JNK2) and impaired TF promoter activation. In contrast, 67LR does not affect the JNK 1/2 independent expression of VCAM-1 in these cells.

Endothelial cells line the vascular lumen while their abluminal surface interacts with the basal membrane. The laminin 10 isoform is the main component of the basal membrane in mature vessels, separating endothelial cells from interstitial collagens [7]. It is notable that the laminin most widely used for in vitro studies was isolated from mouse Engelberth-Holm-Swarm tumors corresponding to the laminin 1 isotype [7]. This form of laminin, however, does not occur in the endothelial cell basement membrane and therefore might constitute a limitation for previous studies. Therefore, the effect of laminin on TNF-α induced TF expression was analysed in this study using commercially available human laminin and mainly containing the laminin 10 isoform.

Separation of endothelial cells from interstitial collagens plays an important role in maintaining vessel wall integrity, since laminin and interstitial collagen modulate endothelial function in a differential manner. Indeed, collagen activates endothelial Src kinase as well as the GTPase Rho while suppressing
the activity of the GTPase Rac, leading to disruption of intercellular junctions, cell proliferation, tube formation, and initiation of angiogenesis [5,6]. In contrast, laminin antagonizes these effects [5,6]. In line with these findings, cells growing on laminin proliferate slower than those on fibronectin. The present study demonstrates that endothelial cells growing on laminin express lower levels of TF upon stimulation with TNF-α as compared to those on fibronectin. This observation is consistent with the interpretation that basal membrane associated laminin preserves vascular integrity by reducing endothelial cell activation.

The interaction between 67LR and the laminin subunit β1 is an essential component of cellular adhesion to the basement membrane [8]. The polyphenol EGCG has been characterized as a ligand for 67LR. Experiments performed in human lung cancer cells demonstrate that EGCG mediated growth inhibition is 67LR dependent and that EGCG binds to the 67LR with a $K_d$ value of 39.9 nM [10]. In endothelial cells, 67LR is involved in shear stress induced endothelial nitric oxide synthase activation, indicating that this receptor has functional properties beyond its known role in cell adherence [9]. The present study demonstrates that 67LR is abundantly expressed on the surface of human aortic endothelial cells and that activation of 67LR by laminin or EGCG inhibits TF protein expression and activity.

TF expression is a major trigger of the thrombotic response, and high levels of TF contribute to thrombus formation following plaque rupture or erosion [4]. The proinflammatory cytokine TNF-α [22] and histamine have been identified as potent inducers of TF expression [2]. Similar to laminin, EGCG
inhibited TNF-α and histamine induced TF expression in a concentration dependent manner. The MAP-kinases JNK1/2, p38, and ERK regulate TF expression at the transcriptional level in response to both agonists [23]. 67LR activation by EGCG resulted in decreased phosphorylation of the MAP kinase JNK1/2 and decreased expression of TF. Conversely, 67LR blockade abolished the inhibitory effects of EGCG on JNK1/2 activation and TF expression. Finally, specific targeting of JNK1/2 with siRNA or the pharmacological inhibitor SP600125 reduced TNF-α induced TF promoter activity, RNA and protein expression. Hence, EGCG inhibits TF expression by decreasing JNK activation.

An involvement of MAP kinases in laminin signalling has been described in tumor cells [24]. To confirm the role of JNK1/2 in mediating the effects of 67LR activation on endothelial TF, TNF-α induced VCAM-1 expression was analyzed, since this protein, is regulated independent of JNK1/2 in this cell type [25]. Neither laminin nor EGCG affected endothelial VCAM-1 expression, confirming that 67LR activation specifically inhibits JNK1/2. It is of note that some previous studies described a role for JNK1/2 in VCAM-1 induction in vascular cells [26;27]. However, it should be considered that different cell types, different experimental protocols, and different SP600125 concentrations may account for these discrepancies. TNF-α induced VCAM-1 expression depends on NFκB activation in endothelial cells [28]. EGCG did not affect IkB-α degradation nor NFκB activation after TNF-α stimulation in HAEC. This result strongly suggests that EGCG inhibits TF expression via a transcription factor other than NFκB and located downstream of JNK1/2, such as AP-1, which is known to regulate TF promoter activation [29].
It is of clinical relevance that the inhibition of TF mRNA and protein expression reached a significant level when EGCG was applied at concentrations as low as 1 and 3 μM. Such levels of EGCG have been measured in human plasma following the oral intake of green tea extract or purified EGCG [30,31]. Thus, the inhibitory effect of 67LR activation by EGCG occurred at clinically relevant concentrations and might account at least in part for the cardioprotective effects of green tea consumption.

In summary, this study demonstrates that 67LR activation by laminin or the polyphenol EGCG inhibits endothelial TF expression and activity. Hence, it describes a new mechanism by which the basement membrane modulates the hemostatic balance in intact vessels and identifies 67LR as a potential target for the development of novel anti-thrombotic therapies.
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**Figure Legends**

**Figure 1: Laminin inhibits TF protein expression**

A. 67LR is abundantly expressed on the surface of HAEC. 20x magnification. B. TNF-α induced TF expression is inhibited when cells are grown on laminin as compared to fibronectin. *p<0.001.

**Figure 2: 67LR inhibits TF protein expression**

EGCG inhibits TNF-α (A) and histamine (B) induced TF protein expression in HAEC. *p<0.001 vs TNF-α and histamine alone. C. EGCG decreases TNF-α induced TF surface activity. *p<0.001 vs TNF-α alone. D. In C57BL6 mice EGCG reduces TF activity. *p<0.05 vs control. E. Treatment with a 67LR blocking antibody (MLUC5) abrogates the inhibitory effect of EGCG on TF expression. *p<0.01 vs TNF-α alone. All blots are normalized to GAPDH.

**Figure 3: 67LR inhibits TF promoter and TF mRNA expression**

A. Real-time PCR analysis demonstrates that EGCG impairs TF mRNA expression in HAEC. Values are normalized to L28 expression. *p<0.0001 vs TNF-α alone. B. Luciferase assay shows that EGCG inhibits TNF-α induced TF promoter activity. Values are normalized to total protein concentration. *p<0.001 vs TNF-α alone.
Figure 4: EGCG impairs JNK1/2 phosphorylation

A. TNF-α induces a transient phosphorylation of the MAP-kinases JNK, ERK, and p38. EGCG inhibits phosphorylation of JNK1/2, but not ERK and p38. No change in total expression of JNK, ERK, and p38 is observed. B. EGCG inhibits activation of JNK, but not ERK and p38. *p<0.01 vs TNF-α alone.

Figure 5: 67LR activation mediates impairment of JNK1/2 activation by EGCG

A. Transfection with siRNA against JNK1 and JNK2 mRNA inhibits TNF-α induced TF expression. *p<0.05 vs TNF-α alone. SP600125, a specific JNK inhibitor, impairs TF protein expression (B) and TF promoter activation (C). *p<0.001 vs TNF-α alone. D. Treatment with a 67LR blocking antibody blunts the inhibitory effect of EGCG on JNK activation. *p<0.001 vs TNF-α alone.

Figure 6: Neither 67LR nor JNK regulate VCAM-1 expression

A. Laminin does not alter TNF-α induced VCAM-1 expression in HAEC as compared to fibronectin. p=NS. B. EGCG has no effect on TNF-α induced VCAM-1 expression. p=NS vs TNF-α alone. C. JNK inhibition with SP600125 does not affect TNF-α induced VCAM-1 expression. p=NS vs TNF-α alone. All blots are normalized to GAPDH.
References


