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Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres



Regular Article

Immunomodulatory drugs increase endothelial tissue factor expression in vitro

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ARTICLE INFO

Article history:

Received 1 April 2010

Received in revised form 17 November 2010

Accepted 17 November 2010

Available online 14 December 2010

ABSTRACT

Introduction: Immunomodulatory compounds such as thalidomide (THL) and lenalidomide (LEN) represent treatment options for multiple myeloma. Venous thromboembolism is a potential complication of immunomodulatory treatment in myeloma patients. The optimal thromboprophylactic strategy to prevent this drug-induced hypercoagulable state is debated. It is the aim of this study to investigate i) the effect of immunomodulatory treatment on endothelial cell tissue factor (TF) expression and function, ii) the regulation of the observed TF activity, and iii) the modulating effect of low molecular weight heparin and aspirin on TF activity in vitro.

Materials and methods: These aims were addressed in an in vitro culture model, human umbilical vein endothelial cells, using TF activity and antigen assays as well flow cytometry, real time PCR and electron microscopy.

Results: At THL and LEN concentrations resembling those observed in myeloma patients in vivo and in the presence of tumor necrosis factor- α (TNF α) we observed significantly increased TF activity in human umbilical vein endothelial cells in vitro. Concordant changes were detected for tissue factor mRNA and TF whole cell antigen. Dalteparin and a mixture of monoclonal anti-TF antibodies inhibited TF activity by 100% and more than 80% respectively, while aspirin's inhibitory effect was only approximately 30%. In the presence of TNF α we detected the generation of endothelial cell-derived microparticles which expressed TF activity.

Conclusions: Our in vitro data support the hypothesis that THL and LEN induce a hypercoagulable state through increased endothelial TF expression.

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Introduction

The immunomodulatory compounds (IMiD), thalidomide (THL) and lenalidomide (LEN), are considered first or second line treatments for multiple myeloma and are being investigated for an expanding range of indications including myelodysplastic syndrome, chronic lymphocytic leukaemia and other B-Cell malignancies [1,2]. Next to immunomodulatory effects, reported mechanisms of THL and LEN therapeutic effects are decreased tumor cell proliferation, decreased tumor cell survival and modified tumor neovascularisation [3,4]. One of the adverse effects of immunomodulatory compounds are venous thromboembolic events (VTE). In myeloma patients under immunomodulatory therapy alone VTE occur in 3–33% depending on the presence of modulating factors such as time in relation to disease diagnosis, tumor burden and co-administered medications such as dexamethasone (DXM) or chemotherapeutic agents [5,6].

The vascular endothelium serves many functions. Endothelial thromboresistance as one of these functions can be defined as the

integration of pro- and anticoagulant properties. Expression of these properties is vascular bed specific and varies in relation to time and to external stimuli. Imbalance or alteration of “differential expression of procoagulants and anticoagulants in the endothelium” may lead to a localized procoagulant phenotype [7,8].

Tissue factor (TF, formerly coagulation factor III (FIII)) is considered to be the most important initiator of coagulation in vivo [9,10]. We hypothesize that when expressed on endothelial cells TF can modulate endothelial thromboresistance from an anticoagulant state into a procoagulant one. Under physiologic conditions endothelial TF expression and activity are believed to be minimal or absent [7]. Our study hypothesis is that immunomodulatory compounds in therapeutic concentrations upregulate endothelial tissue factor expression and activity in the presence of inflammatory stimuli such as tumor necrosis factor α (TNF α) and interleukin-6 (IL6). Both are reportedly present in multiple myeloma [11]. We tested our hypothesis in an in vitro system with human umbilical vein endothelial cells (HUVEC) in culture.

Different prophylactic strategies have been proposed to prevent VTE in patients receiving IMiD treatment including no medical intervention, platelet antiaggregant therapy (aspirin) and anticoagulant therapy (unfractionated heparin or low molecular molecular weight heparins). The efficacy of these strategies is an issue of intense debate as controlled

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prospective studies are lacking. The goal of this study is to elucidate a potential mechanism underlying the hypercoagulable state associated with THL and LEN treatment. For this purpose we investigated i) TF mediated procoagulant effects of endothelial cells in culture (HUVEC) in response to therapeutic concentrations of THL and LEN, ii) the specificity and regulation of the observed TF activity, iii) the effect of low molecular weight heparin, dalteparine (DLT) and aspirin (ASA) on endothelial thromboresistance.

Materials and methods

Reagents

Reagents were purchased from commercial sources or provided by the producer: acetyl-salicylic acid (ASA, Sigma-Aldrich, Buchs, Switzerland), antithrombin (AT, Kybernin P, CLS Bering, Zurich, Switzerland), dalteparin (DLT, Pfizer, Zurich, Switzerland), DMSO (Fluka, Basel, Switzerland), dexamethasone (DXM, Sigma-Aldrich), Endothelial Cell Growth Medium-2 (EGM-2) cell culture media supplemented with EGM-2 bullet kit growth factors and 2% fetal bovine serum (Lonza, Basel, Switzerland), HUVEC (Clonetics: Lonza, Basel, Switzerland) interleukin-6 (IL6, Sigma-Aldrich), triton X-100 (Sigma-Aldrich), tumor necrosis factor- α (TNF α , Sigma-Aldrich).

Handling of immunomodulatory compounds

Due to limited aqueous solubility THL and LEN were solubilized in dimethylsulfoxide (DMSO) at 100 mM and stepwise diluted in the medium at the time of use. The final concentration of DMSO was 0.05% in all experiments as preliminary experiments had shown that DMSO concentrations of 0.5% and higher inhibited the modulation of TF activity induced by THL and LEN (data not shown). THL was purchased at Sigma or donated by Celgene Corporation. LEN was a kind gift of Celgene Corporation. THL and LEN were tested at 5 different concentrations ranging from 0.005 to 50 μ M. The drug concentrations we tested in our experiments (0.005–50 μ M) were in the range of reported maximal steady state plasma levels observed in patients, which are 10.6 μ M for up to 400 mg of THL and 2.2 μ M for 25 mg of LEN (see dashed lines in Fig. 1A) [12,13]. As 0.5 μ M was within the described therapeutic concentration range and proved to be a concentration at which highly reproducible changes were observed for both drugs, further experiments were carried out using this dose.

Cell culture

HUVEC cells were of quality controlled commercial origin and cultured according to the manufacturer's instructions (see also www.lonza.com/go/literature/352). HUVECs were maintained at 37 °C in a humidified incubator in an atmosphere of 5%CO₂/95% air. For the TF activity measurement cells were seeded in 48 well culture plates, for the TF antigen ELISA assay in 75 cm² flasks, and for the TF mRNA RT-PCR measurements in 6 well plates. Cells were seeded at a density of 13000 cells/cm² on day zero, cultured to sub-confluent conditions and experiments were typically performed on day three. This seeding density allowed assessment of the exponential phase of proliferation. All experiments were performed with cells in passage 4–8. All cell culture results shown are based on three individual experiments at least.

Tissue factor activity assay

A commercial TF activity assay (Actichrome TF, American Diagnostica, Stamford, CT) was performed on intact adherent cultured cells and supernatant medium, according to the manufacturer's recommendations, with some modifications as described by Steffel

et al. [14]. Cells were grown in 48-well plates to sub-confluent condition. After incubation with the various reagents, cells were washed with PBS and incubated for 15 minutes with human FVIIa and FX at 37 °C, resulting in the formation of a TF/FVIIa complex on the cell surface. TF/FVIIa complex mediated conversion of human FX to FXa, which was subsequently measured by its ability to cleave a chromogenic substrate. Standard curves were adapted to cover the pM range (0.47–30pM). The lowest value on the standard curve fulfilled the criterion for range of detectability of the assay as defined by the producer ($>$ or $=$ two standard deviations above the mean OD of the "0" standard). In the absence of cytokines, basal TF activity measurements and those for THL and LEN were close to the limit of detectability, absolute differences were small. We thus chose to show only basal TF activity as controls. To verify the specificity of the assay and regulation of TF activity, control experiments were performed using a mixture of anti TF antibodies (TF9-6B4, TF9-9 C3) and a rabbit anti TFPI antibody kindly provided by Dr Wolfram Ruf [15–17].

Tissue factor antigen assay

TF antigen expression was determined on cell lysates, microparticles, and supernatant medium using a commercial ELISA kit (Imubind Tissue Factor, American Diagnostica Inc., Stamford CT). Cell lysates were obtained by lysing cells in phosphate buffered saline (PBS; pH=7.4 containing 1% Triton X-100) for 10 minutes on ice, followed cell scraping and three cycles of freezing/thawing. The ELISA was performed according to the manufacturer's instructions. TF antigen level was expressed in pg/ml using a reference curve created with TF standards provided with the kit. Results are expressed as 10⁻¹² mol/g protein.

Isolation of endothelial microparticles

Cell culture supernatants were collected after 24 h incubation with the various reagents and centrifuged for 10 minutes at 300 g to remove detached cells and debris. Detached cells were resuspended in PBS to perform TF measurements while the remaining supernatant was centrifuged at 17000 g for 30 minutes. The endothelial microparticle (EMP)-rich pellet was resuspended in PBS and centrifuged once more in 17000 g for 30 min for final EMP isolation.

Protein assay

To normalize results of activity and antigen experiments, protein concentration per well or flask was determined using the Mini Bradford methodology following the manufacturers instructions. (Quick Start™ Bradford Protein Assay, BioRad Laboratories, Glattpburg, Switzerland). Results are given in mol/g protein.

Flow cytometry

EMP were analysed by flow cytometry (FC) on a Canto I (BD Biosciences, San Jose, CA) flow cytometer using BD FACSDIVA software (version 5.0.1). Size calibration beads (FC size calibration kit, Molecular Probes, Eugene OR: 1.1, 2.0, 4.2 and 5.9 μ m) were used to characterize the size of the EMP. EMP were quantified absolutely using 50 μ l of quantitation spheres (Flow Count fluorospheres, Beckman Coulter, Mervue, Ireland) of known concentration and of homogenous size (10 μ m nominal diameter; see gate "P7" in Fig. 2E), which were added to the samples. 10000 calibration spheres were counted enabling the calculation of the sample volume that the EMP were analysed in.

Tissue factor real time quantitative reverse-transcriptase PCR

Total mRNA was isolated from endothelial cells using a commercial kit (RNeasy, Minikit, Qiagen, Basel, Switzerland) and quantified

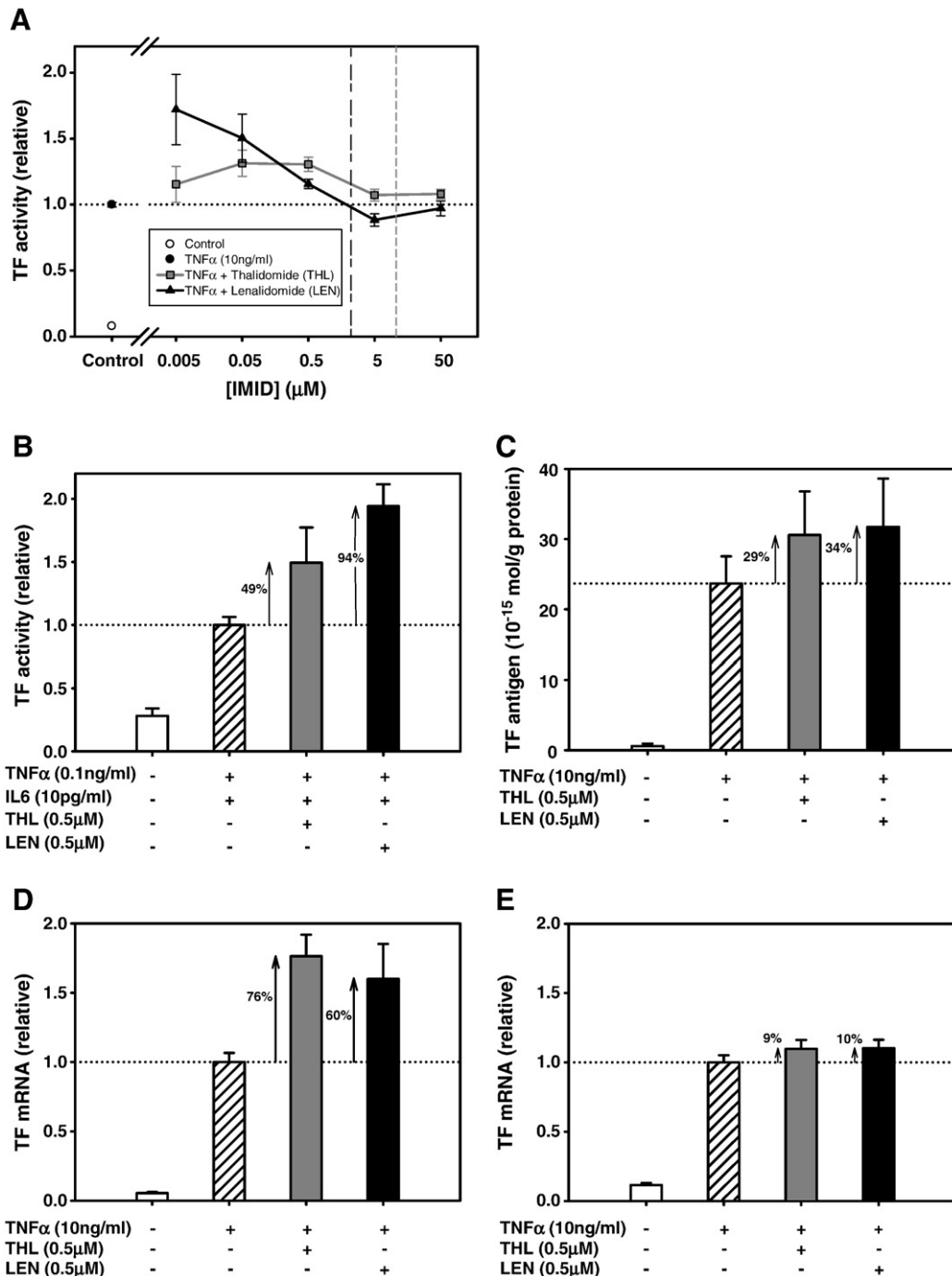


Fig. 1. Effect of immunomodulatory compounds on TF activity and expression. (A) Effect of THL and LEN on TF surface activity of HUVEC exposed to TNF α . HUVEC were incubated for 24 hours with thalidomide (THL; squares) and lenalidomide (LEN; triangles). Dashed lines represent peak concentrations in plasma reported in patients treated with standard therapeutic doses of the respective drug. Results were normalized to TNF α -treated controls (10 ng/ml; dark circle and dotted line); non TNF α -treated controls are given in comparison (light circle). (B) Effect of THL and LEN on TF surface activity in the presence of low-dose TNF α and IL6. TNF α -treated controls (hatched bar) and non cytokine-stimulated cells (white bar) are given in comparison. Incubation time was 24 hours. (C) Effect of THL and LEN on whole-cell TF antigen. TNF α -treated controls (10 ng/ml; hatched bar and dotted line), TNF α /THL (grey bar) and TNF α /LEN (black bar) are depicted. TF antigen was detected by ELISA after lysis of HUVEC cells. Results are given relative to total protein. Incubation time was 24 hours. (D) Effect of THL and LEN on TF mRNA after 1 hour. After one hour of incubation increased levels of TF mRNA levels were observed in TNF α /THL (grey bar; +80%) and TNF α /LEN (black bar; +60%) treated cells relative to TNF α controls (hatched bar). Non TNF α -treated controls are given in white. TF mRNA expression was assayed relative to a low abundance internal control huPO. (E) Effect of THL and LEN on TF mRNA after six hours. After six hours of incubation only minor differences in TF mRNA were observed among the three TNF α -treated experimental groups (TNF α , TNF α /THL, TNF α /LEN). All depicted results stem from a pooled analysis of at least three experiments; error bars represent SEM.

spectrometrically (Nanodrop Technologie LLC, Wilmington, DL). Reverse transcription was carried out according the manufacturers protocol (High capacity cDNA reverse transcription kit 4368814 Applied Biosystems, Foster City, CA). "Single-plex" real-time PCR was performed on an ABI Prism 7700 instrument using TaqMan Fast Universal PCR master mix, specific primers (forward [0.5 μM]; reverse [0.5 μM]) and a

labeled probe [0.25 μM]. Human phosphofructoprotein (ribosomal large human PO or huPO) served as an internal control. 40 cycles (95 $^{\circ}\text{C}$ 3 s, 60 $^{\circ}\text{C}$ 30 s) of real time PCR were carried out in a sample volume of 20 μl . The product was sequenced and found to correspond >95% with the expected sequence. Forward primer: 5'-AAT GTG GAG AGC ACC GGT TCT-3'; reverse primer: 5'-CGT TCA TCT TCT ACG GTC ACA TTC -3';

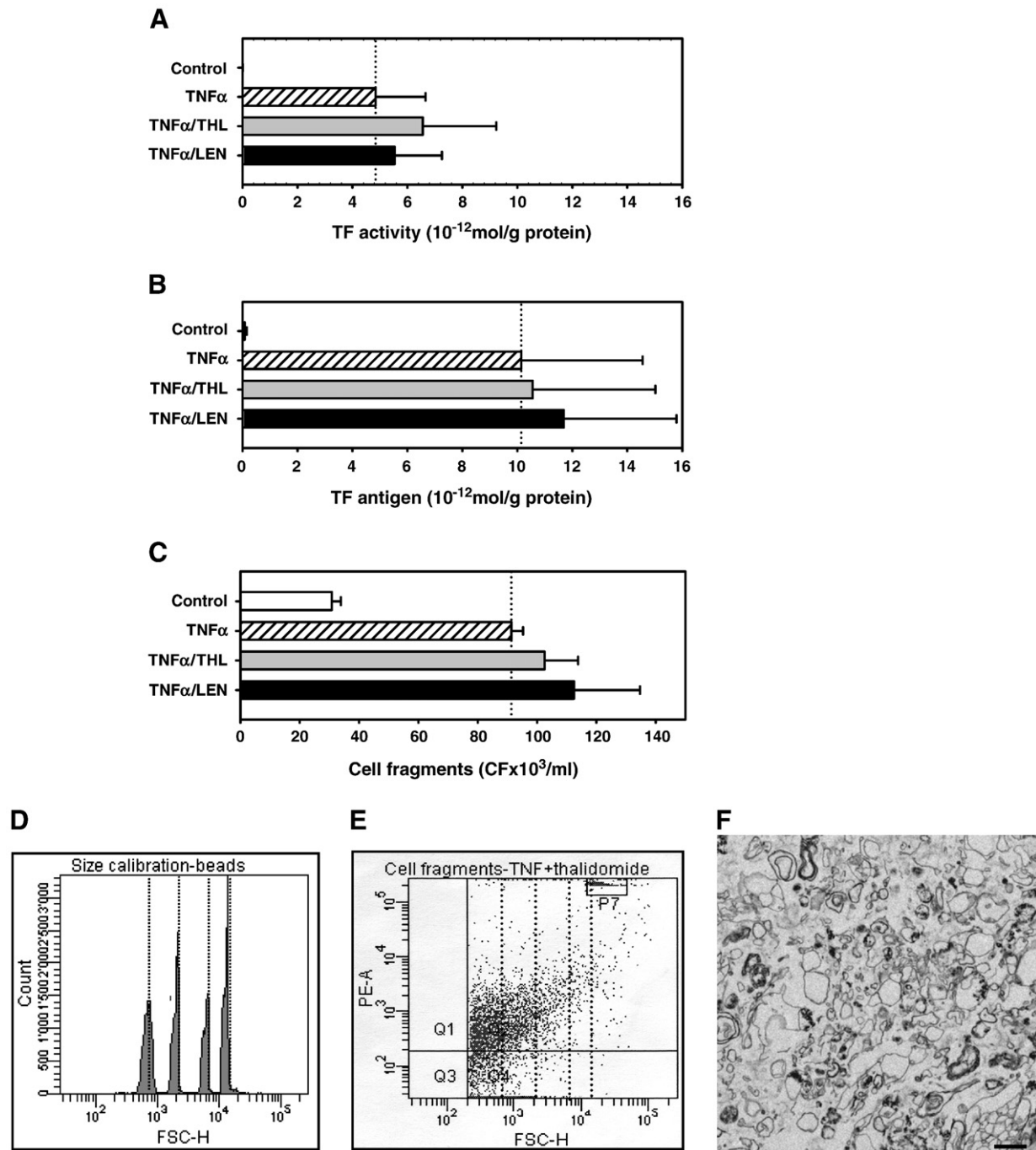


Fig. 2. Endothelial microparticles. Quantitation and characterization of endothelial microparticles (EMP). Supernatant media of HUVEC cells cultured for 24hs were analyzed for endothelial microparticles. (A) EMP-associated TF activity and (B) EMP-associated TF antigen were investigated in these supernatant media. (C) Absolute quantitation was carried out using a known quantity of counting beads added to each sample prior to flow cytometric analysis. For (A) – (C) pooled results of three experiments were shown; horizontal error bars represent SEM. (D) Flow cytometric size calibration. The histograms and corresponding dotted lines represent beads of 1.1, 2, 4 and 6 μ m in diameter. (E) Flow cytometric analysis of EMP. The vertical axis represents fluorescence intensity (phycoerythrin (PE)) for the endothelial marker CD105. The horizontal axis represents forward scatter (FSC). The gate designated P7 at the top right hand corner of image was set to detect counting beads used for absolute quantitation. (F) Transmission electron microscopic image of EMP. Scale bar=1 μ m.

probe: 5'(FAM)-CAC ACCTTA CCT GGA GAC AAA CCT CGG A-3'(TAMRA). Known quantities of internal standard and amplicon cDNA were used to generate standard curves to permit absolute quantitation.

Electron microscopy

Endothelial microparticles were fixed with 2.5% glutaraldehyde in PBS for 30 minutes before the last centrifugation step of the isolation procedure (17000 g for 30 minutes). Subsequently, the microparticles were post fixed with 2% osmium tetroxide in PBS for 30 minutes,

dehydrated in a sequence of ethanol solutions (70% ethanol in water 2 \times 30 min, 96% ethanol in water 30 min, 100% ethanol 30 min, 100% anhydrous ethanol 2 \times 30 min), embedded in Epon (30% Epon in anhydrous ethanol for 1 h, 50% Epon in anhydrous ethanol for 2 h, 70% Epon in anhydrous ethanol over night, 100% Epon for 2 h), and polymerized at 60 $^{\circ}$ C. Thin sections were stained with aqueous uranyl acetate 2% and Reynolds lead citrate and imaged in a Phillips CM 208 transmission electron microscope (FEI, Eindhoven, Netherlands) using a Gatan Bioscan CCD camera (1024 \times 1024 pixels) and digital micrograph acquisition software (Gatan GmbH, Munich, Germany).

Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed using the SPSS for windows version 15.0 (SPSS Inc, Chicago, IL) software, by paired sample t-test or univariate analysis (UNIANOVA) as appropriate. A probability value of <0.05 was considered significant.

Results

Thalidomide and lenalidomide increase TF activity in HUVEC cells

Surface TF procoagulant activity in un-stimulated HUVECs was at the limit of detectability. We were not able to detect a reliable increase in TF procoagulant activity by THL or LEN alone. Treatment of HUVECs with TNF α at 10 ng/ml for 24 hours induced an approximately 10-fold increase in TF activity corresponding to a mean absolute TF concentration for TNF α -treated cells of 23.8×10^{-12} mol/g protein ($n = 27$). To permit pooled analysis the results of at least three experiments were normalized prior to data analysis. Results were compared to the inflammatory baseline level induced by TNF α (Fig. 1). THL up-regulated TF procoagulant activity in all concentrations tested compared to TNF α stimulated cells (Fig. 1A). At a concentration of 0.5 μ M – a concentration that lies within the therapeutic range – THL significantly increased TF surface activity by 30% ($n = 9$, $p < 0.001$). Similarly, LEN increased TF functional expression at concentrations that correspond to human therapeutic plasma levels [13]. At 0.5 μ M LEN induced a TF surface activity up-regulation of 16% compared to TNF α alone ($n = 15$, $p < 0.001$). In view of the dose response characteristics and considerations regarding relevant concentrations in vivo subsequent experiments were performed with the immunomodulatory concentration of 0.5 μ M.

In further activity studies we attempted to imitate conditions witnessed in myeloma patients in vivo by lowering the concentration of TNF α 100-fold to 0.1 ng/ml, which is close to the range of values observed in disease states, and by adding a second cytokine (IL6: 10 pg/ml) at a concentration also within the range observed in vivo [11]. Under this dual cytokine stimulation we could reproduce an immunomodulatory compound-induced increase in TF activity of 49 and 94% respectively (Fig. 1B).

Dexamethasone's (DXM) effect on TF surface activity was tested at three different concentrations (0.1, 1 and 10 μ M; these concentrations cover the range of plasma concentrations of patients treated with up to 40 mg orally qd in the presence of TNF α (10 ng/ml) and in the presence or absence of THL or LEN. In our in vitro model DXM did not up-regulate TF surface activity in either the presence or the absence of THL or LEN (data not shown).

Thalidomide and lenalidomide increase TF whole cell antigen

For the TF antigen assay, HUVECs were stimulated with TNF α (10 ng/ml) and treated with THL (0.5 μ M) or LEN (0.5 μ M) for 24 hours. The TF antigen measurements were performed in endothelial cell lysates. Similarly as in the TF surface activity experiments, both THL and LEN up-regulated TF antigen expression by 29% and 34% respectively compared to TNF α -stimulated cells. TF antigen in unstimulated control cells was very close to the detection limit of the ELISA (Fig. 1C).

Thalidomide and lenalidomide increase TF mRNA

To evaluate whether the observed TF antigen and surface activity up-regulation was due to a possible effect of THL and LEN on TF gene transcription, the levels of TF mRNA stimulated with TNF α (10 ng/ml) and treated with THL (0.5 μ M) or LEN (0.5 μ M) for 1 and 6 hours were evaluated by reverse transcriptase (RT)-PCR. The TF-mRNA analysis showed that control samples expressed very low levels of TF-mRNA

(Fig. 1D and E). Treatment with TNF α markedly increased TF-mRNA levels compared to levels in control cells. Compared to TNF α alone, TNF α /THL and TNF α /LEN increased TF-mRNA levels by 76% and 60% respectively after 1 hour incubation time and by 9% and 10% respectively after 6 hours incubation time (Fig. 1D and E).

Endothelial microparticles

In supernatant media of TNF α , TNF α /THL and TNF α /LEN treated cells there were more endothelial microparticles (EMP) than in medium of control cells (Fig. 2C). TF activity (Fig. 2A) and TF antigen (Fig. 2B) were detected on EMP stemming from cells exposed to TNF α , TNF α /THL and TNF α /LEN, whereas no TF activity or antigen were found on EMP of control cells.

EMP were of variable size ranging from below 1 to more than 6 μ m as indicated by the size calibration beads (Fig. 2E and dotted lines in Fig. 2D) and as demonstrated in the transmission electron micrograph (Fig. 2F). The EMP varied in electron-density: some where of low (depicted) others were of intermediate density (data not shown).

Effect of dalteparin

In order to evaluate the effect of the low molecular weight heparin, DLT, on drug-induced TF up-regulation we first treated HUVECs (cultured in heparin-free medium) with TNF α (10 ng/ml) and THL or LEN for 24 hours and added DLT at 1 IU/ml for the final 1–4 hours of co-incubation. After incubation endothelial cell monolayers were washed with PBS to eliminate any heparin residues before the addition of FVIIa and FX, as a part of the TF activity assay. Under these conditions DLT inhibited TF surface activity induced by TNF α /THL and TNF α /LEN by 25% and 24% respectively. In the presence of IMiDs (THL: grey bars; LEN: black bars), co-incubation of cells with antithrombin (AT) and DLT did not relevantly augment the inhibitory effect induced by DLT alone (Fig. 3A). In the presence of DLT a 12 to 35% reduction of TF activity was observed. Most of this reduction was AT independent.

In a second set of experiments HUVECs (cultured in heparin free medium) were incubated with TNF α and THL or LEN for 24 hours, washed and then treated with DLT (with or without AT), FVIIa and FX for 15 minutes as a part of the TF activity assay (Fig. 3B). This was done in order to investigate the anti-Xa and the antithrombin-dependence of the inhibitory effect of DLT on TF function. In the absence of AT, DLT partially inhibited TF up-regulation by TNF α , TNF α /THL and TNF α /LEN by 32%, 47% and 40% respectively. In the presence of AT, the inhibition of TF activity was total (100%, 100% and 100%).

Effect of aspirin

The effect of aspirin on endothelial TF surface activity was assessed at three different concentrations (0.5, 5 and 50 μ M) in the presence of TNF α (10 ng/ml; Fig. 3C) and also with the further addition of THL (Fig. 3D) or LEN (Fig. 3E). The chosen ASA concentrations correspond to peak plasma concentration observed in patients treated with 20–325 mg of ASA qd [18,19]. In cells stimulated by TNF α alone (Fig. 3C), ASA at concentrations 0.5–5 μ M, inhibited TF activity by 6%, while at 50 μ M TF was up-regulated by 15%. In TNF α /THL treated cells (Fig. 3D) ASA at 0.5, 5 and 50 μ M resulted in 19%, 28% and 31% down-regulation of TF activity respectively. In TNF α /LEN treated cells (Fig. 3E) ASA at 0.5 μ M down-regulated TF by 30%, at 5 μ M did not alter TF activity while at 50 μ M up-regulated TF activity by 6%.

Effect of anti-TF and anti-TFPI antibodies on TF activity

To confirm that factor Xa production measured in the TF activity assay was specific for TF and not mediated by other proteins, TNF α and

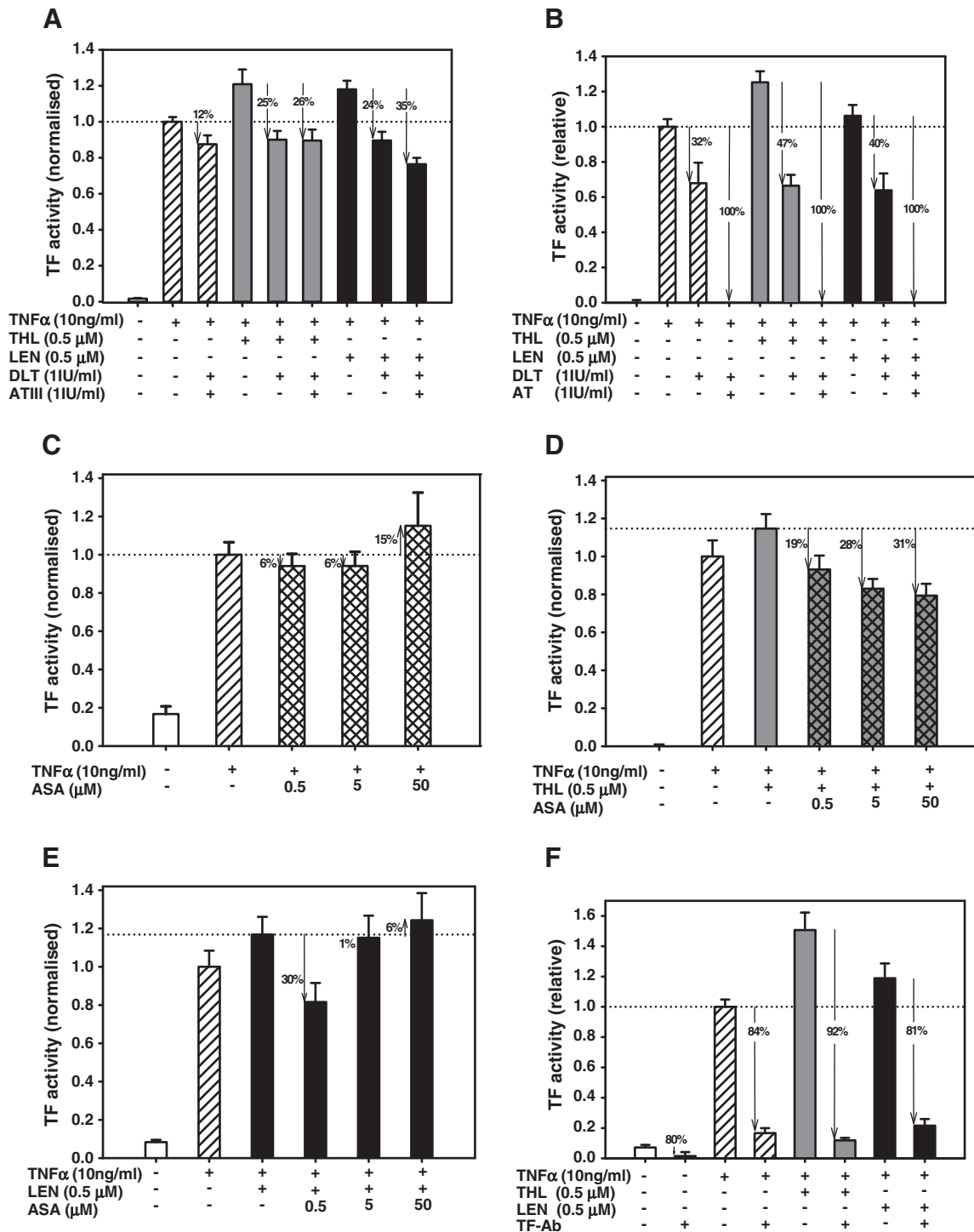


Fig. 3. Effects of dalteparin, aspirin and monoclonal antiTF antibodies. (A) Effect of dalteparin and antithrombin – pre-incubation experiments. Dalteparine (DLT; 1 IU/ml) and antithrombin (AT; 1 IU/ml) were incubated together with TNFα alone, TNFα/THL or TNFα/LEN respectively during the final 1–4 hours of culture in so called pre-incubation experiments. (B) Effect of dalteparin and antithrombin – co-incubation experiments. DLT and AT were co-incubated with the cells during the time of TF activity measurement (15 minutes) in co-incubation experiments. (C) Effect of aspirin alone on TF surface activity. In the presence of TNFα (diagonally hatched bars) only modest modulation of endothelial TF activity was observed in HUVEC incubated in vitro with aspirin (ASA) at concentrations observed in vivo. The white bar indicates the non TNFα-exposed control. (D) Effect of aspirin in combination with thalidomide on TF surface activity. THL-treated cells are depicted as grey bars. ASA exposure is indicated by the cross-hatched pattern. (E) Effect of aspirin in combination with lenalidomide on TF surface activity. (F) Effect of monoclonal TF antibodies on TF surface activity. Cells were exposed for one hour on ice to a cocktail of two monoclonal anti TF antibodies (final concentration 25 μg/ml) known to inhibit TF function. Total incubation time with TNFα, IMiDs and/or ASA was 24 hours for all experiments. All depicted results stem from a pooled analysis of at least three experiments; error bars represent SEM.

THL and LEN pre-treated cells were incubated with an anti-TF antibody mixture for 1 hour on ice. The anti-TF antibody mixture inhibited TF surface activity by 81–92% in the three groups TNFα, TNFα/THL and TNFα/LEN (Fig. 3F).

Addition of an anti-TFPI antibody in our system resulted in an approximately 8 fold increase in TF activity (data not shown). Utilizing similar cell culture models as we did Ahamed et al. and Kushak et al. reported a 5 and 2.8 fold increase, respectively [20,21].

Discussion

Our data show that immunomodulatory drugs (IMiD) increased tissue factor expression and activity *in vitro*. These findings were elaborated utilizing HUVEC in the presence of inflammatory conditions induced by the presence of cytokines (TNF α , IL6). Both cytokines are reported to be increased and of prognostic relevance in conditions including multiple myeloma [11]. TNF α has also been shown to induce TF on non-endothelial cells including monocytes [22]. In the absence of such cytokines endothelial TF expression and activity have been reported to be at or below the level of detectability [7]. Our data confirm the latter findings.

In the presence of TNF α alone (10 ng/ml), THL and LEN at 0.005 to 0.5 μ M increased surface related TF activity by 15 to 72% (study aim i). This increase occurred in a concentration range that corresponds to plasma concentrations observed *in vivo*. Fig. 1A illustrates the increased TF activity for various doses of THL and LEN and also depicts the peak concentrations (dotted vertical lines) reached as reported in the literature [12,13]. The dose-response curves for THL and LEN suggest that there is a concentration for each respective IMiD that is associated with maximum induction of TF activity *in vitro*. Once this concentration is surpassed, TF activity declines (Fig. 1A).

Increased VTE risk has been reported in multiple myeloma (MM) patients treated by high dose DXM [23]. Studies with similar concentrations of DXM as those expected in humans suggested that in our *in vitro* system steroids do not exhibit a direct effect on endothelial TF expression.

To corroborate the activity data and to elucidate the underlying mechanisms we performed TF antigen studies (Fig. 1C) and also assayed TF gene transcription by real time PCR at 1 and 6 h incubation time (Fig. 1D and E). Our results suggest a TNF α dependent, drug-mediated increase in TF gene transcription, which is apparent at 1 hour and by 6 hours has decreased to barely detectable levels. This increase in transcription translates into an increase in whole-cell TF antigen at 24 hours by 29% for THL and 34% for LEN.

Next to increased endothelial cell bound TF our studies also show that TF bearing endothelial derived microparticles are generated in the presence of inflammatory conditions and immunomodulatory compounds (study aim ii). The quantity of TF antigen released by HUVEC in form of precipitable EMP is considerable, as we found approximately one third of the total TF antigen (TF antigen in lysed endothelial cells + TF antigen in lysed EMP) in the EMP. In similar experiments Kushak et al. found 45% of total TF antigen in supernatant media of TNF α treated endothelial cells in culture [21]. EMP bound TF was found to be capable of generating FXa as shown in Fig. 2A. *In vivo*, EMP are considered to be prothrombotic [24]. Our *in vitro* findings thus support a potential role of EMP in immunomodulatory compound-induced hypercoagulability. However, the definition of EMP in our case differs from that of Chironi et al. [24]. We included particles that were larger than 1 μ m as we hypothesized that these may also contribute to hypercoagulability.

In a final series of experiments (study aim iii) we sought to investigate the regulation of the TF response we observed under THL and LEN treatment in the presence of TNF α . Vignoli et al. have previously published data showing that the low molecular weight heparin, Dalteparin, can inhibit TF activity using a similar FXa dependent assay as we employed [25]. Our data confirm these findings and extend them. In experiments where therapeutic concentrations of DLT (1 IU/ml) were added to HUVEC during a 1–4 hour pre-incubation period (Fig. 3A: pre-incubation experiments), an approximately 30% decrease of TF activity was observed. This effect was non antithrombin-dependent.

LMWH prophylaxis has been proposed to prevent VTE in MM patients treated with IMiD [23]. *In vivo* the main anticoagulant effect of heparins, including DLT, is the one mediated by AT [26]. We thus sought to determine if we could observe an AT-dependent or indirect

LMWH effect on HUVEC. Co-incubation experiments were performed where DLT was added to the reagents during the time of FXa dependent TF activity measurement (Fig. 3B). The combination of DLT/AT completely inhibited TF activity – not only to levels of TNF α controls but to those of non TNF α treated cells.

ASA has also been proposed as a VTE prophylactic strategy in MM patients treated with IMiD [23]. *In vitro* and at ASA concentrations corresponding to those observed in patients taking therapeutic doses, we observed a direct effect of ASA on endothelial TF activity in the presence of THL and LEN. The effect was smaller than that observed for LMWH (Fig. 3C–E).

Finally, we could show that drug-induced increased TF activity could be specifically inhibited by a cocktail of anti TF antibodies (Fig. 3F). These findings suggest that TF protein accounts for >80% of the TF activity we observed. This is relevant as others have reported FVIIa binding to another surface protein, endothelial cell protein C receptor, and reduced coagulant activity [27,28]. Our findings correspond with those of Ahamed et al. who observed a >80% reduction in Xa generation in TNF α -treated HUVEC while using another TF activity inhibiting antibody [20].

The main limitation of our study is that it was performed in an *in vitro* system. We furthermore did not evaluate exposure times longer than 24 hours. However, there are preliminary *in vivo* data that appear to lend support to our findings. The Italian Myeloma Network has shown low absolute VTE rates in patients receiving thalidomide containing regimens of 4.5% in enoxaparin-treated patients and 5.5% in ASA treated patients. The observed difference was not statistically significant [29]. In patients with chronic lymphocytic leukemia treated with LEN Aue et al. showed i) a 19% incidence of VTE in LEN treated patients, ii) increased TNF α levels in response to LEN treatment and iii) significantly higher TNF α levels (0.27 vs 0.09 ng/ml) in patients who suffered VTE vs. those who did not [30].

We conclude that immunomodulatory compounds increase endothelial TF expression and activity *in vitro* in a cytokine dependent fashion. Cytokines and immunomodulatory compounds lead to increased endothelial TF synthesis, increased expression and activity of surface bound TF and an increased turnover of TF protein resulting also in the generation of TF-bearing endothelial derived microparticles. *In vitro* DLT inhibits the IMiD-induced reduction of endothelial TF activity more than ASA. We hypothesize that the IMiD-induced procoagulant state of endothelium *in vitro* may give insights into the hypercoagulable state observed *in vivo*.

Conflict of interest statement

Dr Serena Valsami was supported by a travel grant from Celgene permitting her to present data at the International Thrombosis Congress in Athens Greece. The other coauthors, Wolfram Ruf, Maria-Sybille Leikauf, Jerzy Madon, Andres Kaech, and Lars M. Asmis, have no conflict of interest to declare.

Acknowledgements

The tissue factor real time PCR assay, the primers and probes were designed in the course of a project in Dr Jeffrey Rade's research lab at Johns Hopkins Medical Institutions, Baltimore MD in collaboration with Clay Demming. Prof. Jörg Fehr, previous director of the clinic of hematology at the Zurich University Hospital provided research support. THL and LEN were provided by Celgene Corporation, Summit NJ.

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