Accumulation of tissue factor in endothelial cells induces cell apoptosis, mediated through p38 and p53 activation.

Tissue factor-mediated apoptosis

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Keywords: tissue factor, serine-phosphorylation, p38-MAP kinase, p53 activation, apoptosis.

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ABSTRACT

We previously reported that high levels of tissue factor (TF) can induce cellular apoptosis in endothelial. In this study, TF-mediated mechanisms of induction of apoptosis were explored. Endothelial cells were transfected to express wild-type TF. Additionally, cells were transfected to express Asp253-substituted, or Ala253-substitued TF to enhance or prevent TF release respectively. Alternatively, cells were pre-incubated with TF-rich and TF-poor microvesicles. Cell proliferation, apoptosis and the expression of cyclin D1, p53, bax and p21 were measured following activation of cells with PAR2-agonist peptide. Greatest levels of cell proliferation and cyclin D1 expression were observed in cells expressing wild-type or Asp253-substituted TF. In contrast, increased cellular apoptosis was observed in cells expressing Ala253-substituted TF, or cells pre-incubated with TF-rich microvesicles. The level of p53 protein, p53-phosphorylation at ser33, p53 nuclear localisation and transcriptional activity, but not p53 mRNA, were increased in cells expressing wild-type and Ala253-substituted TF, or in cells pre-incubated with TF-rich microvesicles. However, the expression of bax and p21 mRNA, and Bax protein were only increased in cells pre-incubated with TF-rich microvesicle and in cells expressing Ala253-substituted TF. Inhibition of the transcriptional activity of p53 using pifithrin-α suppressed the expression of Bax. Finally, siRNA–mediated suppression of p38α, or inhibition using SB202190 significantly reduced the p53 protein levels, p53 nuclear localisation and transcriptional activity, suppressed Bax expression and prevented cellular apoptosis. In conclusion, accumulation of TF within endothelial cell, or sequestered from the surrounding can induce cellular apoptosis through mechanisms mediated by p38, and involves the stabilisation of p53.

INTRODUCTION

Denudation of endothelial layer occurs as a consequence of endothelial cell apoptosis and is often associated with chronic inflammatory diseases. The association of tissue factor (TF) with apoptotic vascular endothelial cells has long been established [1,2]. Moreover, the release of TF-containing microvesicles by activated endothelial cells has been reported i*n vitro* and *in vivo* [3-6]. The ability of TF to initiate endothelial cell proliferation has previously been demonstrated [7-9]. This function appear to arise from the interaction of TF with integrins [9-14] resulting in the activation of proliferative signalling mechanism including ERK-MAPK pathway [9]. In contrast, at high concentrations of TF, this is accompanied with the arrest of the progression through cell cycle, leading to apoptosis [7] through a mechanism which involves the upregulation of the pro-apoptotic protein Bax. In our previous study [3] we showed that alanine-substitution of serine 253 significantly reduced the release of TF as endothelial cell-derived microvesicles while aspartate-substitution of this residue accelerated the release process. We also observed that the prevention of TF release within cell-derived microvesicles, either through overexpressing an alanine 253-substituted form of TF, or by transfection of the cells with a synthetic peptides analogues to phospho-ser258 form of the cytoplasmic domain of TF, induced cellular apoptosis but only in activated endothelial cells. In addition, we recently reported the prolonged activation of p38 MAP kinase in response to overexpression of wild-type TF in endothelial cells which was further enhanced by the prevention of TF release through alanine 253-substitution [15].

Although there is some evidence that endothelial cells may express TF in response to inflammatory modulators *in vitro* [16-18], the ability of these cells to produce TF *in vivo* remains a subject of debate [19]. Moreover, endothelial cells may acquire and recycle TF carried by circulating microvesicles, or store the TF moiety [19-24]. High levels of TF stores may then be incompatible with the physiological release of TF by endothelial cells.

Consequently, activation of the endothelial cells through injury or inflammation may in turn promote the pro-apoptotic mechanisms which manifest as endothelial dysfunction associated with disease conditions. Recently, there has been a number of studies which report the participation of procoagulant microvesicles from different sources in endothelial apoptosis and dysfunction [25-28] and highlight the importance of the composition of these microvesicles. Moreover. since TF expression is induced as a consequence of alterations in shear stress on endothelial cells [29], hypertensive conditions may also induce the release of TF-rich microvesicles leading to downstream vascular denudation [30]. The aim of the present study was to investigate the mechanisms linking the disruption of TF release with cellular apoptosis using wild-type and mutant forms of TF as tools to manipulate the release of TF. The level and activity of apoptotic modulators was then measured in these cells, and also compared to cells that were pre-incubated with microvesicles containing high and low levels of TF.

MATERIAL AND METHODS

Cell culture, DNA transfection and microvesicle isolation

Human coronary artery endothelial cells (HCAEC) which are isolated primary endothelial cells from healthy bodies (individuals who suffered acute death) and without previous indications of chronic disease or infections, were purchased from PromoCell (Heidelberg, Germany). The cells were cultured in MV media containing 5% (v/v) foetal calf serum (FCS) and growth supplements (PromoCell). The breast cancer cell lines MDA-MB-231 which expresses high levels of TF, and MCF-7 (both cell lines from LGC-ATCC, Teddington, UK) which has negligible TF expression were used throughout to obtain TF-rich and TF-poor microvesicles, respectively. The breast cancer cell lines were cultured in Dulbecco's minimal essential medium containing 10% (v/v) FCS. The cells were adapted to serum-free media and the microvesicles were purified from conditioned media according to

established procedures [3,21]. Additional samples of microvesicles were isolated from the plasma of patients with cardiovascular complications and healthy plasma subjects (Innovative Research, Novi, USA), as previously described [31] and used in particular experiments.

TF was introduced into the cells by two separate means. First, endothelial cells were incubated with TF-poor or TF-rich microvesicles (<0.05 and 14 ng/ml TF) in line with those representing conditions encountered *in vivo* [32,33], and in some cases also with patient and normal microvesicles. Additionally, endothelial cells were transfected to express wild type TF, or mutant forms that are either readily released or strictly retained by cells [3]. The pCMV-XL5-TF plasmid for the expression of full-length human TF was obtained from OriGene (Rockville, USA). Mutant plasmids containing aspartate and alanine substitutions at ser253 were as described before [3]. HCAEC were transfected with 1 μg of plasmid DNA using Lipofectin (Invitrogen, Paisley, UK), in order to express wild-type TF or mutant forms of TF [3]. This procedure permits the attainment of controllable and reproducible high and low levels of TF within the cells [3]. Following transfection, the cells were incubated for 48 h at 37°C to allow the expression of TF. The presence of TF in cells was monitored and was measured to be in line with those previously described for each of the two procedures [3,21]. HCAEC were adapted to serum-free medium (SFM) and activated by incubation with protease activated receptor 2-agonist peptide (PAR2-AP; 20 μM) as described before [3]. The expression of PAR2 and the lack of detectable TF in HCAEC was established previously [3,9]. The microvesicles were characterised as before [21]. Cell number was determined in each sample by staining with crystal violet as previously described [34,35].

Western blot analysis of p53, Cyclin D and Bax proteins and p53 phosphorylation

HCAEC (2×10^5) were seeded out in 12-well plates and transfected to overexpress the wild-type or mutant forms of TF, or EGFP. Alternatively, cells were incubated with high (14 ng/ml) or low (<0.05 ng/ml) levels of TF-containing microvesicles. Samples of cells were

also used untreated. In some experiments, the cells were co-transfected with p38α-siRNA or a control siRNA. Other sets of cells were pre-incubated with either SB202190 (100 nM), pifithrin-α (100 nM) or equivalent amounts of DMSO-vehicle. Cells were activated with PAR2-AP (20 μM) and incubated for 4 to 18 h. The cells were then lysed in Laemmeli's buffer containing a protease inhibitor cocktail (Sigma Chemical Co Ltd, Poole, UK). When determining p53 phosphorylation levels a phosphosafe-lysis buffer was used initially (100 μl; Merck-Millipore, Nottingham, UK). The concentrations of total protein in the samples was determined using Bradford protein-estimation assay and samples (10 μg unless otherwise stated) were separated by 12% (w/v) SDS-PAGE. The protein bands were transferred onto nitrocellulose membranes and blocked with TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20). Total amount of p53 protein was determined by probing the membranes with a rabbit polyclonal anti-human p53 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:1000 (v/v) in TBST. Phosphorylation of p53 at ser33 and ser46 were assessed by probing the membranes with a rabbit polyclonal anti-human p53 (phospho-ser33) antibody (Insight biotechnology, Middlesex, UK) diluted 1:1000 (v/v) in TBST, or a rabbit polyclonal anti-human p53 (phospho-ser46) antibody (New England Biolabs, Hertfordshire, UK) diluted 1:1000 (v/v) in TBST. To avoid discrepancies due to the amount of total p53 in the samples, in these experiments excess amounts were loaded onto the gels. The membranes were then washed with TBST and probed with a goat anti-rabbit alkaline phosphatase-conjugated antibody (Santa Cruz Biotechnology) diluted 1:1000 (v/v), incubated for 90 min. The TF bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega Corp. Southampton, UK) and recorded. To analyse the level of Bax and cyclin D1 proteins, the membranes were probed with a mouse monoclonal anti-human Bax antibody (2D2; Santa Cruz Biotechnology) diluted 1:1000 (v/v) in TBST, or a mouse monoclonal anti-human cyclin D1 (DCS6; New England Biolabs) diluted 1:1000 (v/v) in

TBST. The membranes were then washed with TBST and probed with a goat anti-mouse alkaline phosphatase-conjugated antibody (Santa Cruz Biotechnology) diluted 1:1000 (v/v) and incubated for 90 min. The bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega) and recorded. As loading controls, the level of GAPDH was measured using a goat anti-human GAPDH antibody (V-18; Santa Cruz) followed by a donkey anti-goat alkaline phosphatase-conjugated antibody (Santa Cruz Biotechnology) diluted 1:1000 (v/v) and incubated for 90 min. The TF bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded.

Inhibition of p38 kinase activity, p53 transcriptional activity, and siRNA-mediated knockdown of p38α

In some experiments, the cells were pre-incubated for 30 min with a specific p38 inhibitor SB202190 (100 nM) to inhibit the activity of p38. The inhibition of p38 was previously optimised by measuring the phosphorylation of ATF2 [15]. To suppress the expression of p38α, HCAEC were transfected with p38α-specific siRNA or a control siRNA (Santa Cruz Biotechnology) using Lipofectin (Life Technologies, Paisley, UK). The p38α-specific siRNA consisted of a pool of 4 target-specific 19-25 nt siRNAs designed to knock down gene expression and the optimal concentration of siRNA (45 nM) and the time-point of maximal silencing (48 h) were optimised previously [15]. In some experiments, cells were co-transfected with pCMV-XL5-TF, pCMV-XL5-TF_{Asp253}, pCMV-XL5-TF_{Ala253} or pCMV-EGFP, together with either p38α-siRNA or control-siRNA. To measure the transcriptional activity of p53, cells were co-transfected with 1 µg of Pathdetect p53-Luc *cis*-reporting plasmid (Agilent Technologies, Wokingham, UK), together with TF or EGFP expressing plasmids as above, and incubated for 48 h to permit TF/EGFP protein expression. The cells were then adapted to serum-free medium and activated with PAR2-AP (20 µM) together with one untreated set. The cells were harvested at 8 h, washed and lysed and the luciferase

activity measured using the luciferase measurement system (Promega) as described before [36]. The readings were expressed as compared to a positive control treated with TNFα (10 μg/ml) in each case. In order to suppress the transcriptional activation of p53-responsive genes [37] without influencing non-p53 dependent apoptosis [38,39], the cells were pre-incubated with pifithrin-α (1-(4-Methylphenyl)-2-(4,5,6,7-tetra-hydro-2-imino-3(2*H*)-benzothiazolyl)ethanone hydrobromide) (100 nM; R&D Systems Europe Ltd., Abingdon, UK) for 30 min prior to activation. The effective concentration of pifithrin-α was optimised prior to the experiments, by incubating HCAEC with a range of concentrations of pifithrin-α (0-200 nM) prior to activation using TNFα and measuring the expression of bax protein at 4 h post-activation (Supplemental Fig. I).

Quantification of TF mRNA expression by quantitative real-time RT-PCR

Total RNA was isolated from HCAEC (2×10^5) using the TRI-reagent system (Sigma). Real-time RT-PCR was carried out in triplicates using 100 ng of total RNA from each sample using primer sets designed to amplify p53, cyclin D1, bax, p21 and β-actin mRNA. The amounts of TF mRNA were measured by real-time RT-PCR and the ratios compared to the untreated or control samples, calculated using the $2^{-\Delta\Delta C_T}$ method [40]. The reaction was carried out at an annealing temperature of 60°C using the GoTaq® 1-Step RT-qPCR System (Promega) on an iCycler thermal cycler (Bio-Rad, Hemel Hempstead, UK). The primers used were:

p53-forward: 5'-GTTCCGAGAGCTGAATGAGG-3'

p53-reverse: 5'-TTATGGCGGGAGGTAGACTG-3'

cyclin D1-forward: 5'-CCGTCCATGCGGAAGATC-3'

cyclin D1-reverse: 5'-ATGGCCAGCGGGAAGAC-3'

bax-forward: 5'-CCATCATGGGCTGGACATTGG-3'

bax-reverse: 5'-AGCACTCCCGCCACAAAGATG-3'

p21-forward: 5'-GGAAGACCATGTGGACCTGT-3'

p21-reverse: 5'-GGCGTTTGGAGTGGTAGAAA-3'

β-actin-forward: 5'-TGATGGTGGGCATGGGTCAGA-3'

β-actin-reverse: 5'-GTCGTCCCAGTTGGTGACGAT-3'

Analysis of p53 nuclear localisation by confocal microscopy

HCAEC (3×10^4) were seeded out into glass 35 mm base dishes and transfected to overexpress the wild-type or mutant forms of TF, or alternatively EGFP. Samples of cells were also used untransfected. In some experiments, the cells were co-transfected with p38α-siRNA or a control siRNA as above. Other sets of cells were pre-incubated with either SB202190 (100 nM) or equivalent amounts of DMSO-vehicle. Cells were activated with PAR2-AP (20 μM) and incubated for 6 or 18 h. The cells were then washed three times with PBS (2 ml), fixed with 3 % (v/v) formaldehyde for 30 min, permeabilised using 0.2 % (w/v) Triton X-100 for 4 min and blocked with 5 % (v/v) donkey serum (Sigma) for 30 min. The cells were labelled with a rabbit polyclonal anti-human p53 antibody diluted 1:200 (v/v) in PBS (Santa Cruz Biotechnology) for 2 h at room temperature and then washed a further three times with PBS (2 ml). The cells were then probed with a NorthernLights donkey anti-rabbit IgG-NL637 (R&D system) diluted 1:200 (v/v) in PBS. After three further washes with PBS the nuclei were then labelled by incubation with DAPI (2 μg/ml) (Sigma) for 15 min. All samples were analysed by confocal microscopy at room temperature using a Zeiss LSM 710 confocal microscope with a ×20 objective and images were acquired using the ZEN software (Carl Zeiss Ltd, Welwyn Garden City, UK). Co-localisation coefficients were determined using the ImagePro Plus software (Media Cybernetics, Bethesda, USA). In some cases the change in nuclear localisation was determined by subtracting the coefficients of the samples from that of the untreated sample.

Measurement of cell apoptosis

HCAEC (3×10^4) were seeded out into glass base 35 mm dishes and transfected to overexpress the wild-type or mutant forms of TF. Alternatively, cells were incubated with high (14 ng/ml) or low (<0.05 ng/ml) levels of TF-containing microvesicles for 90 min to permit uptake, as previously described [21]. Other cell samples were also pre-incubated with microvesicles from patient and normal plasma. The cells were then adapted to SFM and stimulated with PAR2-AP (20 μM) for up to 18 h. The cells were fixed using 4% (v/v) formaldehyde and measured using the DeadEnd fluorescence-based TUNEL assay (Promega) and analysed by confocal microscopy at room temperature using a Zeiss LSM 710 confocal microscope with a ×20 objective as above. Due to the fragility of the apoptotic cells flow cytometric quantification was not possible using this technique. Therefore the level of cell apoptosis (5×10^4 cells in 48-well plates) was quantified directly using the TiterTACS™ Colorimetric Apoptosis Detection Kit (AMS Biotechnology Ltd., Abingdon, UK) according to the manufacturers instructions. Other methods of analysis of apoptosis, including measurements of caspase activity and annexin V exposure were not used, since both these events occur when cells are activated and therefore these procedures do not distinguish between cell activation which occurs in response to PAR2 induction, and cellular apoptosis.

Statistical analysis

All data represent the calculated mean values from the number of experiments stated in each figure legend ± the calculated standard error of the mean. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA). One-Way ANOVA procedure was used for the analysis of variance of data against the control with Tukey's honestly significant difference test to highlight statistically significant differences.

RESULTS

Sets of human coronary artery endothelial cells (HCAEC) were transfected to overexpress $TF_{Wt,}$ TF_{Asp253}, TF_{Ala253} or EGFP and the level of expression compared and characterised as before [3]. These cells were compared to untransfected cells in particular experiments and are stated throughout. Alternatively cells were pre-incubated with TF-rich or TF-poor microvesicles for 90 min, to permit accumulation of TF [21]. Unless otherwise stated, no differences between cells expressing EGFP and untransfected cells were detectable. Similarly, cells pre-incubated with TF-poor microvesicles and untreated cells exhibited identical measurable properties. Prior to the study, the effective concentration of pifithrin-α and time-points of maximal cyclin D1 expression and maximal p53 levels were optimised (Supplemental Figs. I-III). In agreement with other published data [41,42], no *de novo* expression of TF was detected following the activation of cells for the duration of these experiments, although induction of TF at later time-points has been reported [43].

PAR2-activation of cells overexpressing TF leads to cyclin D1 upregulation and cell proliferation

Activation of cells with PAR2-AP resulted in increased cell proliferation rate (approximately 11% within 24 h) in cells expressing TF_{Wt}, TF_{Asp253} and to a lower extent in EGFP-expressing cells compared to non-activated cells (Fig. 1A). In contrast, a small reduction in cell proliferation was observed in TF_{Ala253}-expressing cells. Semi-quantitative analysis [40] of cyclin D1 mRNA showed maximal expression to be at 8 h post-induction with PAR2-AP (Supplemental Fig. II). Semi-quantitative RT-PCR analysis showed increases in cyclin D1 mRNA in cells expressing TF_{Wt}, TF_{Asp253} at 8 h post-activation compared to non-activated cells but less in cells expressing TF_{Ala253} (Fig. 1B). This was also reflected in increased Cyclin D protein levels in cells expressing TF_{Wt} and TF_{Asp253} (Figs. 1C&D). Interestingly, although activation of cell expressing TF_{Ala253} induced the upregulation of

cyclin D1 mRNA expression, this did not lead to increased expression of Cyclin D protein, or cell proliferation.

Activation of PAR2 in cells overexpressing TF_{Ala253}, or cells pre-incubated with TF-rich microvesicles induces cellular apoptosis through increased p53 activity and Bax expression

In order to assess the rate of cell apoptosis, cells were transfected to overexpress TF_{Wt}, TF_{Asp253}, TF_{Ala253} or EGFP 48 h prior to testing. Alternatively, cells were incubated with microvesicles containing high levels of TF or deficient in TF. Following activation with PAR2-AP, DNA fragmentation was measured using two different TUNEL-based apoptosis assays, at 24 h post-activation. Due to fragility of the apoptotic cells quantification using flow cytometry, was not feasible at this point. Therefore, qualitative analysis carried out using a fluorescence-based TUNEL assay indicated increased levels of DNA fragmentation in cells expressing TF_{Ala253} (Fig. 2A). Furthermore, these alterations were confirmed using a quantitative TUNEL assay based on HRP-end labelling, and also shown in cells incubated with TF-rich or patient-derived microvesicles (Figs. 2B&C). The differences between the rates of apoptosis, in cells transfected to express EGFP and untransfected cells, or any of the non-activated cells were not significant. Other methods of analysis of apoptosis, including measurements of caspase activity and annexin V exposure were not used since these procedures do not distinguish between cell activation which occurs in response to PAR2 induction, and cellular apoptosis.

To examine the mechanism of the induction of cell apoptosis, the levels of p53 mRNA and protein, the phosphorylation of p53 at ser33 and ser46, and the nuclear localisation of the p53 were analysed. Prior to the experiments, maximal protein level of p53, in response to TNFα (2 μg/ml) was determined to be attained at around 4 h (Supplemental Fig. III). Semi-

quantitative RT-PCR analysis of p53 mRNA levels showed no significant change at 4 h post-activation of cells with PAR2-AP (Fig. 3A) or at any time prior to this (not shown). Furthermore, no significant difference between any of the activated-transfected cell samples was detected (Fig. 3B). In contrast, western blot analysis of p53 protein indicated significantly higher levels of p53 protein at 4 h post-activation, in cells expressing TF_{Wt}, TF_{Asp253} and particularly TF_{Ala253} (Figs. 3C&D). Similarly, pre-incubation of cells with TF-rich microvesicles resulted in increased levels of p53 while pre-incubation with TF-poor microvesicles did not have any significant influence (Figs. 3E&F). Western blot analysis of the phosphorylation state of p53 was carried out using excess amounts of sample to overcome differences in protein quantities and showed increased p53-phosphorylation at ser33, in cells overexpressing TF_{Ala253}, and to a lesser extend in cells expressing TF_{Wt}, following activation (Figs. 4A&B) but phosphorylation of ser46 was barely detectable in any of the samples (Supplemental Fig. IV). In addition, pre-incubation of cells with TF-rich microvesicles resulted in increased phosphorylation of p53 at ser33, while pre-incubation with TF-poor microvesicles did not have any significant influence (Figs. 4C&D). Furthermore, analysis of the nuclear localisation of p53 using confocal microscopy indicated the accumulation of p53 within the nucleus, in cells overexpressing TF_{Wt} and TF_{Ala253} following activation of PAR2 (Fig. 4E & Fig. 5). Finally, analysis of the p53-mediated expression using a luciferase-reporter vector demonstrated enhanced transcriptional activity of p53, in cells overexpressing TF_{Ala253} following activation of PAR2 (Fig. 4F). As a more representative indicator, the pre-incubation of cells with patient-derived microvesicles, but not normal plasma microvesicles also resulted in increased phosphorylation of serine 33 on p53 (Figs. 4G&H).

Activation of PAR2 in cells transfected to overexpress TF_{Ala253}, or cells pre-incubated with TF-rich microvesicles resulted in the upregulation of both bax mRNA at 4 h post-activation (Figs. 6A&B). Furthermore, the expression of Bax protein was increased in at 18 h

post-activation in cells transfected to overexpress TF_{Ala253} (Figs. 6C&D), cells pre-incubated with TF-rich microvesicles (Figs. 6E&F), or cells pre-incubated with patient-derived microvesicles (Figs. 6G&H). The expression of p21 mRNA was also increased in cells expressing TF_{Ala253} at 4 h post-activation (Fig. 6I). Moreover, pre-incubation of the cells with an inhibitor of the transcriptional activity of p53 (pifithrin-α) suppressed the upregulation of bax mRNA (Fig. 7A) and prevented apoptosis in PAR2-AP activated cells, expressing TF_{Ala253} (Fig. 7B). Pifithrin-α is known to prevent cell apoptosis specifically through inhibiting the transcriptional activity of p53 [37] and without affecting other mechanisms involved in apoptosis [38,39].

Inhibition of p38 suppress the induction of apoptosis in cell overexpressing TF_{Ala253}

HCAEC were transfected to overexpress TF_{Wt}, TF_{Ala253} or EGFP, together with a specific siRNA to suppress p38α or a control siRNA. Alternatively, the cells were pre-incubated with SB202190 (100 nM) to specifically inhibit p38 activity [15]. The cells were activated with PAR2-AP as before. Inhibition of p38 significantly reduced cellular apoptosis in cells expressing TF_{Ala253} while restoring the level of apoptosis in cells expressing TF_{Wt} (Fig. 8A). siRNA-mediated suppression of p38α expression in cells expressing TF_{Wt}, TF_{Ala253} or EGFP significantly reduced the amount of p53 protein compared similar samples co-transfected with the control siRNA (Fig. 8B). Measurement of p53 phosphorylation by western blot analysis also showed the reduction in the phosphorylation of ser33 in cells expressing TF_{Ala253} (Figs. 8C&D) which was also achievable by inhibition of p38 using SB202190 (Fig. 4F). These were accompanied with lower nuclear localisation (Fig. 5 & Fig. 8E) and p53 transcriptional activity in TF_{Ala253}-expressing cells (Fig. 8F). Finally the expression of Cyclin D in cell samples expressing TF_{Ala253} increased following suppression of p38α (Figs. 8G&H).

DISCUSSION

We previously showed that while incubation of endothelial cells [7,9] and cardiomyocyte cell line H9c2 [44] with moderate amounts of recombinant or microvesicle-derived TF results in cell proliferation, higher concentrations of TF, representative of those observed in severe disease can cause cellular apoptosis [7,44]. In addition, it has been reported that the prolonged exposure of HUVEC to microvesicles obtained from activated THP-1 cells can result in apoptosis [45]. Our previous data indicated that the disruption of TF release into microvesicles can also lead to increased levels of apoptosis in endothelial cells [3]. In our studies, the concentration of overexpressed TF was in line with, but lower than that observed in TNFα-treated endothelial cells and lower than in cells treated with IL-1β [3]. Therefore it is likely that the activated endothelial cells would be capable of releasing sufficient amounts of wild-type TF to prevent apoptosis. However, protein components of microvesicles may accumulate within endothelial cells by uptake of microvesicles [20-22]. In order to use a consistent model during the current study, we examined these mechanisms in cells overexpressing alanine 253-substituted TF which is not released by the cells, and compared these to cells expressing wild-type TF and aspartate 253-substituted TF. These were also compared to cells that were pre-incubated with TF-rich or TF-poor microvesicles [9,21]. Consistent with our previous data, activation of cells resulted in increased cyclin D1 expression and entry into the cell cycle (Fig. 1). However, increased cell apoptosis was only observable in cells expressing TF_{Ala253} or cells incubated with TF-rich microvesicles (Fig. 2). Therefore, it is plausible that the entry into cell cycle, following PAR2 activation, is enhanced by the presence of TF. However, the prolonged presence of TF and/or inability of the cell to release TF within microvesicles results in cell cycle arrest. Our previous evidence

suggests that the arrest of cells on incubation of cells with high concentrations of TF occurs within the G1-checkpoint [7].

One feature of PAR2 activation in TF-expressing endothelial cells is the prolonged phosphorylation of p38 in cells expressing TF_{Wt} which is augmented in cells expressing TF_{Ala253} but is absent in cells expressing TF_{Asp253} [15]. In addition, we have shown that ser258 within the cytoplasmic domain of TF is itself a target for p38α activity [15]. p38 is also capable of phosphorylating ser33 and ser46 within the trans-activation domain of p53 protein following various stress signals to the cell [46-48]. Phosphorylation of ser33 and/or ser46 are pre-requisite for the stabilization of p53 within the nucleus, by dissociating its negative regulator, Mdm2. The resultant induction of p53-mediated transcription initiates the upregulation of the expression of number of genes including p21 [49,50] and bax [50] which lead to cell cycle arrest and apoptosis, respectively. Our data showed an increase in p53 protein but not mRNA, in cells expressing either the wild-type or mutant forms of TF (Figs. 3A-F) suggesting that the increase was due to stabilization of p53 rather than *de novo* expression. This was concurrent with increased p38-dependent phosphorylation of p53 at ser33 but not ser46 in these samples (Figs. 4A-D & Figs. 8C&D). However, the regulation of p53 activity may differ according to cell type and the stimulus [51] and therefore, phosphorylation of ser33 and ser46 within p53 may occur via different mechanisms [52] and independent of other residues [48,53]. The phosphorylation of ser33 is mediated by p38 [46,48] while ser46 may also be phosphorylated by protein kinase Cδ [54]. Interestingly, the serine-proline-leucine motif is present around both ser258 within TF (KENSPLNVS) and ser33 within p53 (NVLSPLPSQ) which further agrees with our finding that the phosphorylation of ser258 within TF is mediated by p38α [15]. However, despite the increased nuclear localisation of p53 in cells expressing TF_{Wt} or TF_{Ala253}, p53 transcriptional activity was only observed in cells expressing TF_{Ala253} and resulted in increased expression of

Bax and p21 proteins in these cells, leading to cellular apoptosis. This observation alludes to a mechanism by which the transcriptional activity of p53 is restrained in samples able to release TF. The prevention of apoptosis through the downregulation of p53 activity by serine/threonine-phosphatases has previously been demonstrated [55,56]. Moreover, following the phosphorylation of ser33, the action of peptidyl-prolyl isolmerase 1 (Pin1) protects p53 from de-phosphorylation, acting as a delay mechanism and enhancing the promotion of cell apoptosis [57,58]. Interestingly, this protection does not alter the amount of p53 protein but preserves the transcriptional activity of p53 [59]. Such a mechanism of protection explains why despite the increased levels and nuclear localisation of p53 in cells expressing TF_{Wt} no increase in Bax or cell apoptosis was observed in these cells. Furthermore, due to the similarity between the sequences surrounding ser33 in p53 and ser258 in TF, it is likely that Pin1 also protects the phosphorylation state of TF against phosphatase activity. This feedback mechanism may preserve the phosphorylation state of TF which may in turn permit the further release of wild type TF, but not TF_{Ala253} and is currently under investigation in our laboratory. The TF-mediated up-regulation of Bax and the induction of cell apoptosis appear to be solely dependent on the transcriptional activity of p53 and were abrogated by pre-incubation of cells with pifithrin-α (Figs. 7A&B). Therefore, the mechanism of TF-induced cell apoptosis appears to be mediated through the intrinsic pathway of apoptosis although our previous data suggest some cross-talk to the extrinsic pathway of apoptosis [44]. Therefore, in agreement with our previous reports, the activity of p38, following PAR2 activation of TF-bearing cells, may have a bi-functional outcome resulting in the induction of cell proliferation, or alternatively cell apoptosis depending on the duration and magnitude of p38 activity.

The activation of cells in response to injury, trauma or inflammatory mediators results in the release of TF as cell-derived microvesicles. At lower concentrations of TF, cells respond

to injury in the form of increased proliferation. However, accumulation of large amounts of TF through high levels of pro-inflammatory factors, or through the sequestration of microvesicles from the bloodstream may compromise the ability of endothelial cells to release TF efficiently, resulting in induction of apoptosis. In conclusion, this study has shown that induction of cellular apoptosis by TF is mediated through the prolonged activation of p38, leading to the phosphorylation of ser33 and stabilization of the transcriptional activity of p53, localised within the nucleus. This in turn results in the expression of Bax and induction of cell apoptosis.

ACKNOWLEDGEMENTS

The support from the Hull and East Riding Cardiac Trust Fund is acknowledged.

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Figure Legends

Figure 1 Induction of cell proliferation and Cyclin D expression in HCAEC expressing TF variants. HCAEC (2×10^5) expressing TF_{Wt} TF_{Asp253}, TF_{Ala253} or EGFP were adapted to SFM and activated with PAR2-AP (20 µM). An untransfected/non-activated sample was included for comparison. A) cell numbers were determined as a percentage of control cells using crystal violet staining (n=3, *=$p<0.05$ vs untransfected/non-activated sample; **=$p<0.05$ vs activated cell sample expressing TF_{Wt}). B) Total RNA was isolated from the cells at 8 h post-activation and the relative amount of cyclin D1 mRNA was determined by RT-PCR (n=3, *=$p<0.05$ vs respective non-activated sample). C) The amount of Cyclin D protein was determined by western blot analysis and D) quantified against GAPDH (n=3, *=$p<0.05$ vs untransfected/non-activated sample).

Figure 2 Analysis of cellular apoptosis in HCAEC expressing TF variants. A) HCAEC (3×10^4) were seeded out into 35 mm glass-base dishes and transfected to express TF_{Wt} TF_{Asp253}, TF_{Ala253} for 48 h, or used untransfected. The cells were adapted to SFM and activated with PAR2-AP (20 µM) for 18 h. The cells were then fixed, developed using the DeadEnd TUNEL assay and analysed by confocal microscopy with a ×20 objective. B) HCAEC (5×10^4) were seeded out into 48-well dishes and transfected to express TF_{Wt} TF_{Asp253}, TF_{Ala253} or EGFP for 48 h. The cells were adapted to SFM and activated with PAR2-AP (20 µM) for 18 h. The level of cell apoptosis was quantified using the TiterTACS™ Apoptosis Detection Kit. (n=3, *=$p<0.05$ vs EGFP-expressing sample). C) HCAEC (5×10^4) were seeded out into 48-well dishes and incubated with TF-rich and TF-poor microvesicles, as well as microvesicles derived from patient and normal plasma, for 90 min. Samples of cells were also treated with TNFα (10 ng/ml) or left untreated and used as positive and negative controls. The cells were adapted to SFM and activated with PAR2-AP

(20 µM) for 18 h. The level of cell apoptosis was quantified using the TiterTACS™ Apoptosis Detection Kit. (n=3, *=p<0.05 vs untreated sample).

Figure 3. Analysis of the influence of expression of TF on p53 expression. HCAEC (2×10^5) expressing TF_{Wt}, TF_{Ala253} or EGFP were adapted to SFM and activated with PAR2-AP (20 µM). An untransfected/non-activated sample was included. Total RNA was isolated at 4 h post-activation and the relative amount of p53 mRNA A) in non-activated and activated cell samples, and B) in activated cells expressing TF_{Wt}, TF_{Ala253} and EGFP was determined. C) The amount of p53 protein was analysed at 4 h post-activation by western blot and D) quantified against GAPDH (n=3, *=p<0.05 vs untransfected/non-treated sample). HCAEC (2×10^5) were pre-incubated with TF-rich or TF-poor microvesicles or left untreated. The cells were adapted to SFM and activated with PAR2-AP (20 µM). E) The amount of p53 protein was analysed at 4 h post-activation by western blot and F) quantified against GAPDH (n=3, *=p<0.05 vs untreated sample).

Figure 4. Analysis of the influence of expression of TF on p53 function. HCAEC (2×10^5) were transfected to express TF_{Wt}, TF_{Ala253} or EGFP. Sets were treated with SB202190 (100nM) or used untreated. The samples were adapted to SFM and activated with PAR2-AP (20 µM). Untransfected/non-activated sample were also included. A) The level of ser33-phosphorylation in the transfected cells was measured using an anti-phosphoser33 p53 antibody and B) normalised against the total p53 protein (n=3, *=p<0.05 vs EGFP-expressing sample without SB202190; **=p<0.05 vs respective untreated sample). HCAEC (2×10^5) were pre-incubated with TF-rich or TF-poor microvesicles. An untreated sample was also included. The samples were adapted to SFM and activated with PAR2-AP (20 µM). C) The level of ser33-phosphorylation in cells pre-incubated with TF-rich or TF-poor microvesicles,

was measured using an anti-phosphoser33 p53 antibody and D) normalised against the total p53 protein (n=3, *=p<0.05 vs untreated sample). E) HCAEC (3×10^4) were seeded out into glass 35 mm base dishes, transfected to express TF_{Wt}, TF_{Asp253}, TF_{Ala253} or EGFP. Samples of cells were also used untransfected. The samples were activated with PAR2-AP (20 μM). Additional cells were either treated with TNFα (10 ng/ml) or used untreated. The cells were then fixed, permeabilised and labelled with anti-human p53 antibody developed with a NorthernLights-donkey anti-rabbit IgG-NL637. The nuclei were then labelled with DAPI and analysed by confocal microscopy with a ×20 objective (Also see Fig. 5). Co-localisation coefficients were determined using the ImagePro Plus software and the change in localisation calculated (n=3, *=p<0.05 vs untransfected/non-treated sample). F) HCAEC (2×10^5) were co-transfected to express TF_{Wt} TF_{Asp253}, TF_{Ala253} or EGFP expressing plasmids, together with p53-Luc plasmid and incubated for 48 h. The cells were adapted to SFM and activated with PAR2-AP (20 μM). The cells were harvested 8 h, washed and luciferase activity measured as a percentage of the positive a sample treated with TNFα (10 μg/ml) (n=3, *=p<0.05 vs cells containing the reporter only; **=p<0.05 vs cell sample expressing TF_{Wt}). G) In addition, ser33-phosphorylation in cells pre-incubated with microvesicles derived from patient and normal plasma, was measured using an anti-phospho-ser33 p53 antibody and H) normalised against the total p53 protein (n=3, *=p<0.05 vs untreated sample).

Figure 5 Analysis of nuclear localisation of p53 in activated HCAEC, expressing TF variants. HCAEC (3×10^4) were seeded out into 35 mm glass base dishes, transfected TF_{Wt}, TF_{Asp253}, TF_{Ala253} or used untransfected, and activated with PAR2-AP (20 μM). Sets of cells were also either treated with TNFα (10 ng/ml) or were not activated. Additionally, a set of cells expressing TF_{Ala253} was incubated with SB202190 (100 nM). All cells were incubated for 4 h. The cells were then fixed, permeabilised and labelled with a rabbit anti-human p53

antibody and probed with a NorthernLights donkey anti-rabbit IgG-NL637. The nuclei were labelled with DAPI and analysed by confocal microscopy with a ×20 objective.

Figure 6 Induction of Bax and p21 expression in HCAEC expressing TF variants or pre-incubated with microvesicles. A) HCAEC (2×10^5) expressing TF_{Wt}, TF_{Asp253}, TF_{Ala253} or EGFP were adapted to SFM and activated with PAR2-AP (20 µM). An untransfected/non-activated sample was included. Total RNA was isolated from the cells at 8 h post-activation and the relative amount of bax mRNA was determined by RT-PCR (n=3, *=$p<0.05$ vs untransfected/non-treated sample). B) HCAEC (2×10^5) were also pre-incubated with TF-rich or TF-poor microvesicles adapted to SFM and activated with PAR2-AP (20 µM), or used without activation. Total RNA was isolated from the cells at 8 h post-activation and the relative amount of bax mRNA was determined by RT-PCR (n=3, *=$p<0.05$ vs untreated sample). C) The amount of Bax protein in the transfected cells was determined by western blot analysis and D) quantified against GAPDH (n=3, *=$p<0.05$ vs EGFP-expressing cells). E) The amount of Bax protein in the pre-incubated cells was also determined by western blot analysis and F) quantified against GAPDH (n=3, *=$p<0.05$ vs untreated sample). G) Additionally, the amount of Bax protein in cells, pre-incubated with microvesicles derived from patient and normal plasma cells was also determined by western blot analysis and H) quantified against GAPDH (n=3, *=$p<0.05$ vs untreated sample). I) HCAEC (2×10^5) expressing TF_{Wt}, TF_{Asp253}, TF_{Ala253} or EGFP were adapted to SFM and activated with PAR2-AP (20 µM). An untransfected/non-activated sample was included. Total RNA was isolated from the cells at 4 h post-activation and the relative amount of p21 mRNA was determined by RT-PCR (n=3, *=$p<0.05$ vs untransfected/non-treated sample).

Figure 7 The influence of pifithrin-α on the expression of bax and cellular apoptosis. A) HCAEC (2×10^5) were transfected to express TF_{Wt}, TF_{Ala253} or EGFP but pre-incubated with pifithrin-α (100 nM) prior to activation and the level of bax mRNA compared to untreated samples (n=3, *=p<0.05 vs untransfected/non-treated sample; **=p<0.05 vs respective sample without pifithrin-α). B) HCAEC (5×10^4 cells) were transfected to express TF_{Wt}, TF_{Ala253} or EGFP and pre-incubated with pifithrin-α (100 nM) prior to activation. Apoptosis was measured using the Apoptosis Detection Kit. (n=3, *=p<0.05 vs an EGFP-expressing cells).

Figure 8 Induction of expression in HCAEC expressing TF variants. HCAEC (5×10^4) expressing TF_{Wt}, TF_{Ala253} or EGFP and were pre-incubated with, or without SB202190 (100 nM) for 30 min. Both sets of cells were then activated with PAR2-AP (20 µM) and apoptosis was measured at 18 h using the Apoptosis Detection Kit (n=3, *=p<0.05 vs untransfected/non-treated sample; **=p<0.05 vs respective untreated sample). B) HCAEC (2×10^5) were transfected to express TF_{Wt} but co-transfected with either p38 siRNA or a control siRNA. The cells were then adapted to SFM and activated with PAR2-AP (20 µM). An untransfected/non-activated sample was included for comparison. The amount of p53 protein at 4 h post-activation was determined by western blot analysis and quantified against GAPDH (n=3, *=p<0.05 vs respective control-siRNA sample). C) The level of phosphorylation of ser33 was measured using an anti-phosphoserine-33 p53 antibody and D) normalised against the total p53 protein (n=3, *=p<0.05 vs sample with control siRNA). E) HCAEC (3×10^4) were seeded out into glass 35 mm base dishes and transfected to express TF_{Wt} but co-transfected with either p38 siRNA or a control siRNA. Cells were activated with PAR2-AP (20 µM) and incubated for 4 h. The cells were then fixed, permeabilised and labelled with anti-human p53 antibody developed with a NorthernLights donkey anti-rabbit

IgG-NL637. The nuclei were then labelled with DAPI and analysed by confocal microscopy with a ×20 objective. Co-localisation coefficients were determined using the ImagePro Plus software (n=3, *=p<0.05 vs untransfected/non-treated sample). F) HCAEC (2×10^5) were co-transfected to express TF_{Ala253} together with p53-Luc plasmid. The cells were adapted SFM and activated with PAR2-AP (20 μM) together with an untreated set. The cells were harvested 8 h, washed and luciferase activity measured as a percentage of the positive a sample treated with TNFα (10 μg/ml) (n=3, *=p<0.05 vs non-treated sample expressing TF_{Ala253}). G) HCAEC (2×10^5) were transfected to express TF_{Ala253} but co-transfected with either p38 siRNA or a control siRNA. The cells were then adapted to SFM and activated with PAR2-AP (20 μM). The amount of Cyclin D protein was determined by western blot analysis and H) quantified against GAPDH (n=3, *=p<0.05 vs sample treated with control siRNA).