Comparison of tissue factor expression and activity in foetal and adult endothelial cells

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Short title: Tissue factor expression in endothelial cells

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Abstract

Objectives: Tissue factor (TF) is not usually expressed by endothelial cells but can be induced in these cells by inflammatory cytokines. Many studies have used human umbilical vein endothelial cells (HUVEC) as a model to examine the regulation of TF expression. However, there is a question as to whether this reflects TF expression in adult endothelial cells. This study compared TF expression and the release of TF-positive microvesicles (MVs) in HUVEC and adult human dermal blood endothelial cells (HDBEC) in response to tumour necrosis factor alpha (TNF α) and interleukin-1 beta (IL-1 β). **Methods:** Cells were treated with the inflammatory cytokines and TF mRNA and total protein expression was examined by real-time RT-PCR and TF-ELISA. Cell surface TF activity was measured in the Calibrated Automated Thrombogram (CAT) assay, as were MV concentrations and MV-associated TF activity. The TF antigen content of the MVs was determined by TF-ELISA. **Results:** Both HUVEC and HDBEC expressed increased levels of TF mRNA in response to TNF α and IL-1 β within 2 h. TF antigen expression increased in both cell types, reaching a maximum at 6 h, with HUVEC expressing significantly higher levels compared to HDBEC in response to TNF α . However, increases in TF-specific thrombin generation were similar on both HUVEC and HDBEC and both cell types also released comparable levels of TF-positive-MVs. **Conclusions:** HUVEC and HDBEC respond similarly to TNF α and IL-1 β in terms of TF expression, and both are suitable models to examine cell surface TF activity and TF-positive MV release in endothelial cells.

Key words: Endothelial cells; tissue factor; procoagulant activity; inflammatory cytokines; microvesicles

Introduction

The endothelium has key roles in processes including angiogenesis, inflammatory response, vasoregulation and haemostasis. Tissue factor (TF) is a 47 kDa transmembrane protein that initiates the extrinsic coagulation cascade by binding to circulating coagulation factor VII/ VIIa (FVII/FVIIa). TF is not normally expressed by cells in contact with the blood such as vascular endothelial cells and leukocytes. However, TF expression can be induced in endothelial cells by inflammatory cytokines such as tumour necrosis factor alpha (TNF α) [1, 2, 3] and interleukin-1 beta (IL-1 β) [4, 5]. The vascular endothelium plays an essential role in the regulation of haemostasis by providing an anti-coagulant surface under normal physiological conditions which can rapidly switch to a procoagulant surface when required to limit blood loss. However, aberrant expression of TF and unregulated activation of coagulation in disease states can precipitate thrombosis. Disease-mediated inflammation can induce coagulation through the upregulation of TF in response to inflammatory cytokines [6, 7], and has been implicated in the pathogenesis of chronic disease [8, 9]. TF can also be released into the circulation in the form of endothelial cell-derived microvesicles (MVs) [10, 11]. TF-positive MVs have been shown to promote thrombosis in animal models [12, 13], and increased levels of procoagulant endothelial cell-derived MVs have been detected in the circulation of patients with chronic diseases such as acute coronary syndrome [14, 15], diabetes [16] and renal disease [17].

Due to the importance of TF expression in vascular physiology and pathological conditions many studies have examined the expression of TF in endothelial cells. Human umbilical vein endothelial cells (HUVEC) are commonly used as an *in vitro* model of endothelial cells to examine regulators of TF expression. However, since these

endothelial cells are derived from foetal tissue, it may be inaccurate to translate data from experiments employing HUVEC to adult vascular diseases such as atherosclerosis. Therefore, in this study we have examined the hypothesis that foetal endothelial cells are more sensitive to inflammatory cytokines than adult endothelial cells, and therefore express higher levels of TF following stimulation with cytokines. Microvascular endothelial cells are also being used more frequently for *in vitro* studies as their commercial availability increases. Furthermore, studies that have detected TF expression on endothelial cells *in vivo* have usually detected TF specifically on microvascular endothelial cells [18, 19, 20]. Therefore, we have compared the expression of TF mRNA and protein, as well as cell surface TF activity and the release of TF into MVs in HUVEC and adult microvascular endothelial cells.

Methods

Cell Culture

HUVEC (PromoCell, Heidelberg, Germany) and microvascular adult human dermal blood endothelial cells (HDBEC) from adult skin (PromoCell) were cultured in endothelial cell growth medium MV (PromoCell) supplemented with 5 % (v/v) foetal calf serum (FCS) and endothelial cell medium MV growth supplements (endothelial cell growth supplement (0.004 mg/ml), recombinant human epidermal growth factor (10 ng/ml), heparin (90 µg/ml) and hydrocortisone (1 µg/ml)). Cells were cultured at 37°C under 5 % (v/v) CO₂ and used between passages 2 and 7. Cells were seeded out into appropriate plates or T75 flasks at specific densities to allow for 80-90 % confluence prior to incubation with inflammatory cytokines. On the day of the experiments, cells

were placed in endothelial cell growth medium MV containing 1 % (v/v) FCS and stimulated with recombinant TNF α (Thermo Fisher Scientific, Paisley, UK) (10 ng/ml) or recombinant IL-1 β (Millipore, Hertfordshire, UK) (10 ng/ml) for various times depending on whether TF mRNA, protein, activity or MV release was being measured.

Real-time RT-PCR

Cells (2×10^5) were seeded out into 6-well plates 24 h before treatment. Cells were incubated with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) for 2-4 h and then lysed in 1 ml of TRIzol reagent (Sigma-Aldrich, Dorset, UK). Chloroform (200 μ l) was added to each sample, vortexed and centrifuged at 10,000 g for 15 min on a microcentrifuge. The clear aqueous phase was transferred to a fresh 1.5 ml tube and RNA was precipitated by the addition of isopropanol (500 μ l) and incubated at room temperature for 10 min. The samples were centrifuged again, the RNA pellet washed with ethanol (75 % (v/v)) and then resuspended in RNase-free water. DNA was removed from the samples by incubation with recombinant DNase I (Sigma) (15 U) for 15 min at room temperature. Stop solution (50 mM EDTA) was then added to the samples and incubated at 70°C for 10 min to inactivate the DNase. Real-time RT-PCR was carried out using the GoTaq 1-step RT-qPCR system (Promega, Southampton, UK) on a ViiA 7 Real-Time PCR machine (Thermo Fisher Scientific) using 82 ng of RNA and specific primers for TF and β -actin (Qiagen, Hilden, Germany). RNA was also isolated from MDA-MB-231 cells, which constitutively express TF and 82 ng of MDA-MB-231 RNA was run alongside the endothelial RNA samples in all RT-PCR assays as a positive control for TF mRNA expression. The RT step was carried out at 37°C for 15 min followed by

inactivation of the reverse transcriptase at 95°C for 10 min. This was followed by 40 cycles of PCR with an annealing temperature of 55°C for 30 s, elongation at 72°C for 30 s and DNA denaturation at 95°C for 30 s. No-template controls and no reverse transcriptase controls were included in all experiments. Ct values were calculated using the ViiA 7 software. Amplified samples were examined by agarose gel electrophoresis to confirm one specific product was generated using the TF or β -actin primers. The efficiencies of the primer sets were calculated to be 89 % for the TF primers and 92 % for the β -actin primers. The ratio of TF mRNA expression in endothelial cells was normalised to β -actin for each sample, and was also normalised to a sample of RNA from MDA-MB-231 cells.

TF ELISA

Cells (2×10^5) were seeded out into 6-well plates 24 h before treatment. Cells were incubated with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) and then lysed at 2, 4, 6, 16, 20, or 24 h in 250 μ l of cell lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100) supplemented with 1 % protease inhibitor cocktail (P8340; Sigma). Briefly, cells were incubated on ice for 30 min, passed four times through a syringe (2 ml) and cell debris was removed by centrifugation at 10,000 g for 10 min at 4°C on a microcentrifuge. The Tissue Factor Quantikine ELISA kit (Bio-Techne Ltd, Abingdon, UK) was used to quantify TF protein levels in the cell lysate samples. Cell lysates were diluted 1:1 in diluent buffer and the ELISA was carried out according to the manufacturer's instructions.

Cell surface TF activity assay

The cell surface TF-specific procoagulant activity of intact, adherent endothelial cells was determined using a TF activity assay [21] with some modifications. Endothelial cells (3×10^4) were seeded out into 96-well plates and incubated at 37°C for 24 h. The cells were then placed in endothelial cell growth medium MV containing 1 % (v/v) FCS and stimulated with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) for 5 h. The cells were then washed twice with 200 μ l of HEPES buffered saline (HBS) (20 mM HEPES pH 7.4, 140 mM NaCl) containing 1 mg/ml BSA and 0.1 % (v/v) sodium azide, and incubated with either the inhibitory TF antibody HTF1 (20 μ g/ml) to block TF procoagulant activity (eBioscience, Cheshire, UK) or mouse control IgG (20 μ g/ml) for 15 min at room temperature. Platelet-poor human pooled plasma from 15 donors (ethical approval obtained from the University of Leicester) taken into sodium citrate tubes and supplemented with corn trypsin inhibitor (CTI) (18.3 μ g/ml) (Cambridge Biosciences, Cambridge, UK) was filtered through a 0.22 μ m filter and 60 μ l was added to each well. Thrombin generation was determined by calibrated automated thrombography (CAT) over 60 min at 37°C on a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific). Data was analysed using the Thrombinoscope software. Lag times and endogenous thrombin potential (ETP) were obtained for each sample. ETP represents the total amount of thrombin generated following the initiation of coagulation by TF in the CAT assay. TF-specific ETP was calculated as the ETP of samples incubated with control IgG minus the ETP of samples with the same treatment but incubated with the inhibitory TF antibody HTF1.

Microvesicle isolation and analysis

Endothelial cells were cultured in T75 flasks until 80-90 % confluent. Cells were placed in endothelial cell growth medium MV (10 ml) containing 1 % (v/v) 0.22 μ m-filtered FCS, and were treated with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) for 5 h or 18 h. The medium was then centrifuged at 1500 g for 15 min to remove cell debris, followed by centrifugation at 40,000 g for 30 min at 20°C to pellet the MVs. The MVs were then washed with HEPES buffered saline, pH 7.4 (HBS) (8 ml), centrifuged again, resuspended in HBS (100 μ l) and stored at -80°C. The concentrations of MVs and the TF activity of the MVs were determined using the CAT assay as described above with some modifications. The cell-derived MV samples were mixed at a ratio of 1:4 with 0.22 μ m-filtered platelet-poor pooled human plasma containing CTI (18.3 μ g/ml). The MP reagent (4 μ M phospholipids, no TF) (Diagnostica Stago Ltd, Ontario, Canada) was used to measure the TF activity of the MVs, whereas the PRP reagent (no phospholipids, 1 pM TF) was used to measure the phospholipid-mediated procoagulant activity of the MV samples. 20 μ l of the MP or PRP reagent was added per well in 96-well plates followed by the addition of the MV/human plasma mix (80 μ l). Thrombin generation was then measured using the CAT assay on a Fluoroskan Ascent fluorometer. The TF antigen content of the MVs was measured using the Quantikine TF-ELISA. Triton X-100 (1 % final concentration) was added to the MV samples and vortexed to free TF from the lipid vesicles into solution. Samples were then analysed using the TF-ELISA as described above.

Apoptosis assay

Endothelial cells (1.5×10^4) were seeded out into 96-well plates 24 h before experiments, placed in endothelial cell growth medium containing 1 % FCS, and treated with TNF α (10 ng/ml) or IL-1 β (10 ng/ml) for 18 h. Apoptosis was assessed by measuring DNA fragmentation using the HT TiterTACS assay kit (Trevigen, MD, US) according to the manufacturers' instructions. The total number of cells per well was then determined by crystal violet staining as before [22]. The ratio of 450 nm to 595 nm was calculated to determine the percentage of apoptotic cells for each sample.

Statistical analysis

Data represent the calculated mean values from the number of experiments stated in the figure legends $\pm$ the calculated standard error of the mean. The data were analysed using the statistical package for the social sciences (SPSS). Significance was determined using one-way ANOVA and the Tukey's honestly significance test. Values of $p < 0.05$ were considered to be significant.

Results

TNF α and IL-1 β both induce expression of TF mRNA, protein and activity in both endothelial cell types

Neither type of endothelial cell expressed detectable amounts of TF mRNA without stimulation, but following stimulation of both HUVEC and HDBEC with either TNF α or IL-1 β , TF mRNA expression was induced within 2 h (Figure 1). Generally HUVEC appeared to express a higher level of TF mRNA compared to HDBEC. TF mRNA expression was followed by the expression of TF protein, with maximal expression observed at 6 h post-treatment, returning to near-basal levels by 24 h (Figures 2A&B). HUVEC expressed significantly higher levels of TF compared to HDBEC following incubation with TNF α at 4-6 h (Figure 2A). In addition, stimulation of both cell types with IL-1 β resulted in higher levels of TF protein expression compared to treatment with TNF α (Figure 2B).

TF activity on the surface of endothelial cells following treatment with TNF α or IL-1 β was measured using the CAT assay which allows endogenous thrombin potential and lag times to be determined. TF-mediated thrombin generation was measured in the absence of any exogenous reagents but in the presence of the inhibitory anti-TF antibody HTF1 to block TF procoagulant activity, or control IgG. TF-specific thrombin generation was then determined by subtracting the cellular thrombin generation in the presence of the inhibitory anti-TF antibody from the uninhibited total thrombin generation activity of the cells incubated with control IgG. Treatment of both HUVEC and HDBEC with either TNF α or IL-1 β resulted in similar increases in cell surface TF-specific procoagulant activity which was demonstrated by increases in TF-specific thrombin generation (Figure 3A). This was reflected in the significant reduction in lag times in both cell types following stimulation with either cytokine (Figure 3B).

Both endothelial cell types release TF positive MVs in response to TNF α and IL-1 β

In the following experiments cells were treated with TNF α or IL-1 β and MVs were isolated from the conditioned media at 5 h and 18 h post-treatment. Analysis of the MVs using a TF-ELISA revealed similar increases in the concentration of TF antigen associated with MVs from both HUVEC and HDBEC (Figure 4A). Additionally, while low levels of TF were released following stimulation with TNF α or IL-1 β for 5 h (Figure 4A), significantly higher amounts of TF were released by 18 h. TF activity of the endothelial cell-derived MVs was determined using the CAT assay with the MP reagent which has excess phospholipids but no TF, and therefore determines levels of TF activity. Examination of isolated MVs revealed similar decreases in lag times associated with MVs derived from HUVEC and HDBEC stimulated with TNF α or IL-1 β at 5 h post-treatment, which become shorter and more significant at 18 h post-treatment (Figure 4B), indicating the incorporation of TF within the released MVs. Identical aliquots of the isolated MVs were also measured using the PRP reagent which contains TF but no phospholipids in order to determine the levels of PS-positive MVs. Examination of thrombin generation showed similar increases in levels of PS-positive MVs released from the two endothelial cell types in response to TNF α and IL-1 β (Figure 4C).

TF expression and release of TF-positive MVs by endothelial cells in response to TNF α and IL-1 β is not due to apoptosis

To establish whether the expression of TF and release of MVs from the cells was due to cell activation or apoptosis, the level of DNA fragmentation in the cells was measured using a TUNEL (TACS) assay at 18 h post-treatment. No increases in DNA fragmentation were observed following treatment with $\text{TNF}\alpha$ or $\text{IL-1}\beta$ for 18 h, in either endothelial cell type (Figure 5). This compared with high numbers of DNA breaks detected by the TACS assay in the positive control cells, which were incubated with nuclease for 30 min (Figure 5). This shows that $\text{TNF}\alpha$ and $\text{IL-1}\beta$ did not induce apoptosis in the endothelial cells, and also indicates that TF expression and TF-positive MV release were not due to apoptosis.

Discussion

It is well known that mediators such as inflammatory cytokines [1-5], lipopolysaccharide (LPS) [23, 24, 25], vascular endothelial growth factor [26, 27] and thrombin [3, 28] induce the expression of TF in endothelial cells *in vitro*. Many studies have used HUVEC as a model of endothelial cells to examine modulators of TF expression. However, HUVEC are derived from foetal tissue and may respond differently to adult endothelial cells. There is also controversy as to whether adult endothelial cells can express TF because some *in vivo* [29] and *in vitro* studies [30] have been unable to detect TF expression in these cells. Furthermore, although HUVEC have been shown to express TF in response to LPS *in vitro* [23, 24, 25], deletion of the TF gene specifically in endothelial cells had no effect on thrombin generation in a mouse model of endotoxemia [31], suggesting that endothelial cells do not express TF during this process *in vivo*. In cases where TF has been detected on endothelial cells *in vivo* this

has been limited to specific vascular beds, usually on microvascular endothelial cells [18, 19, 20], or has been postulated to be as a result of the transfer of TF from leukocytes to endothelial cells [32]. Therefore, in this study we examined the hypothesis that foetal endothelial cells more readily express TF in response to inflammatory cytokines, whereas TF expression is more tightly regulated in adult endothelial cells.

In agreement with previous reports [1-5], both HUVEC and HDBEC expressed increased levels of TF mRNA and antigen in response to $\text{TNF}\alpha$ and $\text{IL-1}\beta$. There was a trend for HUVEC to express higher levels of TF mRNA than HDBEC (Figure 1) and HUVEC also expressed significantly higher levels of total TF antigen compared to HDBEC in response to $\text{TNF}\alpha$ (Figure 2). This is consistent with a previous study in which neonatal microvascular endothelial cells were found to express higher levels of TF mRNA, antigen and activity in response to $\text{IL-1}\alpha$ than the same type of microvascular endothelial cells from adult donors [33]. Interestingly, this was shown to be partly due to the increased sensitivity of neonatal endothelial cells to activate the p38 mitogen-activated protein kinase signalling pathway in response to inflammatory cytokines [33]. Furthermore, a study by Tan *et al.* [34] showed that HUVEC are more sensitive to inflammatory cytokines than adult saphenous vein endothelial cells, with HUVEC expressing higher levels of adhesion molecules in response to $\text{TNF}\alpha$ and $\text{IL-1}\beta$. Together with the present study, these findings support the hypothesis that neonatal endothelial cells are more sensitive to inflammatory cytokines, and more readily upregulate TF expression compared to adult endothelial cells. The fact that both Buzby *et al.* [33] and the present study demonstrated that neonatal endothelial cells express higher levels of TF than adult endothelial cells is intriguing because TF expression is required for developmental angiogenesis [35], and new blood vessels in close proximity

to tumours also express TF on vascular endothelial cells [20]. In contrast Grabowski *et al.* [36] found no difference in the levels of TF mRNA and antigen between TNF α and IL-1 α stimulated HUVEC and adult human saphenous vein endothelial cells, but did detect higher levels of TF-dependent FXa generation in HUVEC compared to adult endothelial cells following stimulation with inflammatory cytokines. In the present study, greater increases in TF mRNA and protein expression were observed in neonatal HUVEC compared to adult microvascular endothelial cells, but this was not reflected in levels of cell surface TF procoagulant activity, which were increased to similar levels in both HUVEC and HDBEC following incubation with TNF α or IL-1 β . These studies demonstrate the differences in TF expression that exist between different types of endothelial cells, and highlight the importance of selecting the appropriate type of endothelial cell or using a variety of endothelial cell types, depending on the question being addressed.

The increases in TF antigen expression observed in HUVEC compared to HDBEC following incubation with TNF α and IL-1 β were not reflected in levels of cell surface TF-specific thrombin generation; a finding that was similar in both cell types. This disparity between levels of total TF antigen expression and cell surface TF activity levels in HUVEC may be an ability of HUVEC to retain TF intracellularly to prevent unregulated coagulation. In fact, a previous study has shown that a combination of TNF α and VEGF is required for enhanced cell surface TF expression and activity on HUVEC compared to TNF α alone [37]. Furthermore, a study by Solberg *et al.* [30] did not detect FXa generation following incubation of adult human saphenous vein endothelial cells with thrombin or endotoxin. However this may be due to differences in how TF activity is measured in different studies. Both Solberg *et al.* [30] and the present

study measured the TF activity of intact endothelial cells, whereas Buzby *et al.* [33] measured the TF activity of cells lysed by freeze-thawing, a process which may enhance TF activity.

Endothelial cells release MVs in response to inflammatory stimuli [38], and increased levels of circulating endothelial-derived MVs have been detected in patients with acute coronary syndrome [14,15, 39], type 1 diabetes [40] and renal disease [17,41].

Endothelial cell-derived MVs are therefore an important biomarker of vascular injury and disease [42]. In this study, both HUVEC and HDBEC released similar levels of TF-positive MVs in response to TNF α and IL-1 β (Figure 4). Since MVs bud directly from the cell surface and incorporate cell surface antigens, this reflects the similar levels of TF activity detected on the surface of HUVEC and HDBEC in response to inflammatory cytokines (Figure 3).

It should be noted that there are additional differences between the two cell types used in this study other than they are derived from foetal or adult tissue. The cells examined in this study are derived from different vascular beds; HUVEC are macrovascular whereas HDBEC are microvascular. It must also be appreciated that endothelial cells taken out of their normal tissue environment will change because they are no longer receiving signals from the extracellular matrix and other surrounding cell types [43, 44] and may therefore not respond in the same way to stimuli as they do *in vivo*. This may explain discrepancies between TF expression observed in *in vitro* models and *in vivo* [30]. The ability to culture endothelial cells from different vascular beds and the range of commercially available human endothelial cells have greatly increased in recent years and it would be of interest to compare the expression of TF in other types of endothelial cells.

Conclusions

This study has demonstrated that HUVEC and HDBEC express similar levels of cell surface TF activity and release similar amounts of TF-positive MVs in response to inflammatory cytokines, but HUVEC express higher total levels of TF antigen in response to TNF α . Therefore while HUVEC may be suitable to examine modulators of TF expression *in vitro*, it may be advisable to study other relevant endothelial cells alongside that better reflect the disease state being examined.

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Figure 1. TF mRNA expression in HUVEC and HDBEC in response to TNF α and IL-1 β .

Endothelial cells were placed in low serum (1 % FCS) endothelial cell growth medium and incubated with 10 ng/ml TNF α (n=3) (A) or 10 ng/ml IL-1 β (n=4) (B) for 0, 2 and 4 h. RNA was isolated using the TRIzol method and TF mRNA was amplified by real-time RT-PCR and normalised to β -actin. TF: β -actin ratios were then normalised to values obtained with MDA-MB-231 mRNA.

Figure 2. Total TF protein expression in HUVEC and HDBEC in response to TNF α and IL-1 β .

Endothelial cells were placed in low serum (1 % FCS) endothelial cell growth medium and incubated with TNF α (A) or IL-1 β (B) (10 ng/ml) for 0-24 h. Cells were lysed and TF protein expression was determined using a TF-ELISA. n=3-5, * = p<0.008, ** = p<0.004, *** = p0.0001 vs. respective untreated controls. # = p 0.036 vs. HUVEC at 4 h, ## = p 0.01 vs. HUVEC at 6 h.

Figure 3. Cell surface TF activity of HUVEC and HDBEC following incubation with TNF α and IL-1 β .

Endothelial cells in 96-well plates were placed in low serum (1 % FCS) endothelial cell growth medium and incubated with TNF α or IL-1 β (10 ng/ml) for 5 h. Cells were

incubated with the anti-TF inhibitory antibody HTF1 or control IgG (20 µg/ml) for 15 min. Filtered human pooled plasma was then added to each well and thrombin generation and lag times were measured by the CAT assay. A) TF-specific endogenous thrombin potential, n=4, * = p 0.007 vs. untreated HDBEC. B) Lag times, n=6, * = p 0.0001 vs. untreated control.

Figure 4. TF-MV release from HUVEC and HDBEC in response to TNFα and IL-1β. Endothelial cells in T75 flasks were placed in low serum (1 % FCS) endothelial cell growth medium and incubated with TNFα or IL-1β (10 ng/ml). Following 5 h or 18 h incubation, MVs were isolated by centrifugation. A) The TF antigen content of the MVs was measured using the TF-ELISA, n=3, * = p0.001 vs. untreated control. ** = p0.03 vs. HUVEC treated with IL-1β. B) TF activity of the MVs following 5 h incubation (n=3, * = p 0.0001 vs. untreated HUVEC, ** = p0.027 vs. untreated HDBEC) and 18 h (n=3, * = p 0.014 vs. untreated HUVEC, ** = p0.008 vs. untreated HUVEC, *** = p0.037 vs. untreated HDBEC) was measured using the CAT assay with the MV reagent. C) Following 5 and 18 h incubation, levels of PS-positive MVs were determined using the CAT assay with the PRP reagent (n=3, * = p0.047 vs. untreated HUVEC).

Figure 5. HUVEC and HDBEC apoptosis following incubation with TNFα and IL-1β. Endothelial cells were placed in low serum (1 % FCS) endothelial cell growth medium and incubated with TNFα or IL-1β (10 ng/ml) for 18 h. A) DNA fragmentation was assessed using the HT TiterTACS colourimetric assay. Negative control samples were incubated without the TdT enzyme, whereas positive control samples were

incubated with nuclease to generate DNA breaks. The total number of cells was determined by crystal violet staining and the ratio of total number of cells (595 nm) to apoptosis measurement (450 nm) was determined. $n=3$, $* = p<0.0001$.