Low molecular weight heparin downregulates tissue factor expression and activity by modulating growth factor receptor-mediated induction of nuclear factor-κB

Camille Ettelaie\textsuperscript{a}, Donna Fountain\textsuperscript{a}, Mary Elizabeth W Collier\textsuperscript{a}, Azza M ElKeeb\textsuperscript{b}, Yu Pei Xiao\textsuperscript{b}, Anthony Maraveyas\textsuperscript{b}

\textsuperscript{a}Biomedical Section, Department of Biological Sciences and \textsuperscript{b}Division of Cancer, Postgraduate Medical Institute in association with Hull York Medical School, University of Hull, Cottingham Road, Hull, HU6 7RX

Correspondence to Dr Camille Ettelaie, Biomedical Section, Department of Biological Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

Email: C.Ettelaie@hull.ac.uk

Tel: +44(0)1482-465528

Fax: +44(0)1482-465458
Abstract

Treatment of cancer patients with low molecular weight heparin (LMWH) appears to have beneficial effects. In this study, the influence of low molecular weight heparin (LMWH) on tissue factor (TF) expression and activity in five cell lines from various tissues was analysed and explored. Incubation of cells with LMWH (0-2000 µg/ml) resulted in the downregulation of TF mRNA expression which was both LMWH concentration-dependent and time-dependent. Downregulation of TF was also measured as decreased cellular TF antigen and activity. Consistently, incubation of cells with LMWH suppressed the nuclear localisation and the transcriptional activity of NFκB. Decreased TF mRNA was largely achievable by incubating the cells with an NFκB inhibitor alone while incubation with betulinic acid to activate NFκB reversed the inhibitory influence of LMWH. Cells were also incubated with a range of concentrations of EGF (0-10 ng/ml), bFGF (0-20 ng/ml) or VEGF (0-4 ng/ml) in the presence or absence of LMWH (200 µg/ml) for 24 h and TF antigen measured. Inclusion of LMWH reduced TF expression in response to EGF, bFGF or VEGF but TF expression was partially restored by increasing concentrations of the growth factors. We conclude that LMWH downregulates TF expression in vitro through a mechanism that involves interference with the function of growth factors which in turn is mediated through the downregulation of the transcriptional activity of NFκB. This mechanism may also explain some of the beneficial influences attributed to LMWH therapy in the treatment of cancer patients.

Keywords: tissue factor, low molecular weight heparin, NFκB, growth factor receptor, cancer cell lines

Abbreviations: TF: tissue factor, LMWH: low molecular weight heparin, NFκB: nuclear factor κB, EGF: epidermal growth factor, bFGF: basic fibroblast growth factor, VEGF: vascular endothelial growth factor
1. Introduction

The association between increased tissue factor (TF) expression and thrombotic complications in cancer is well established [1], and the expression of TF by tumour cells is suggested to be an early event during the onset of the disease [2]. High levels of TF activity have been shown to increase the risk of thrombotic events in cancer patients [3-7] and in particular, the release of TF as circulating microparticles is reported to be an important link between cancer and thrombosis [8-12]. Heparin treatment of cancer patients has been reported to be beneficial in the control of the hypercoagulable state [13]. In addition to the anticoagulant effect, heparin-compounds have been associated with the reduction in the cellular procoagulant activity [14-16]. In particular, low molecular weight heparin (LMWH) is thought to be effective in the regulation of the procoagulant activity [17]. Similarly, we previously reported the reduction in levels of circulating TF in the plasma from pancreatic cancer patients receiving prophylactic LMWH, compared to those not receiving the treatment [18]. The majority of the data reporting the influence of LMWH treatment on TF expression has been acquired from in vivo measurements carried out in cancer patients. Therefore, due to the heterogeneous nature of the samples, any possible specificity of the action of LMWH on downregulating TF expression is undetermined. In order to examine the influence of LMWH in a homogenous population of cells, in this study we have investigated the influence of LMWH on TF expression and activity in cancer cell lines from five separate tissues; pancreatic, breast, colocarcinoma, ovarian and melanoma. We have quantified the TF mRNA and protein, as well as the surface TF activity in these cells. In addition, we have identified the transcriptional activity and cellular localisation of nuclear factor-κB (NFκB) as the likely mechanism by which LMWH downregulates TF expression. The regulation of TF expression by NFκB has previously been reported [19] and it is known that heparin exerts an anti-inflammatory influence on cells [20-24]. The mechanism by which LMWH suppresses the
transcriptional activity of NFκB is thought to involve interference with the nuclear translocation of NFκB [22-24]. Finally, by incubating the cell lines with a range of concentrations of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in the presence and absence of LMWH, we have postulated an underlying mechanism by which LMWH prevents the induction of TF expression, by suppressing the growth factor receptor-mediated activation of NFκB.

2. Material and Methods

2.1. Cell culture

The pancreatic cell line BxPC-3 (LGC-ATCC, Teddington, UK) was cultured in RPMI-1640 medium; MDA-MB-231 breast cancer cell line was cultured in Leibovitz's L-15 medium; LoVo colorectal carcinoma cell line was cultured in F-12K medium; SKOV-3 ovarian cancer cell line was cultured in McCoy's 5a medium and A375 malignant melanoma cell line was cultured in Dulbecco's Modified Eagle's medium. All preparations of media contained 10% (v/v) foetal calf serum (FCS) and 1% (v/v) antibiotic/antimycotic solution. The expression and activity of TF in these cells was confirmed as described below. Cells were supplemented with a range of LMWH concentrations (Mr ~3,000) (Sigma Chemical Company Ltd, Poole, UK) containing approximately 56 units/mg of heparin activity and confirmed using dalteparin at equivalent activities (units/ml).

2.2. Preparation of standard TF mRNA and measurement of TF mRNA expression by quantitative real-time RT-PCR

To prepare a TF mRNA standard, full-length TF cDNA was cloned into the pT7T3-18 vector and used to express the target mRNA. The plasmid was digested with Sac I (10 units, Promega Corporation, Southampton, UK) at 37°C for 1 h and the 3’-overhang was filled in
with T4-DNA polymerase (9.7 units/ml, Promega). TF mRNA was transcribed using the MAXIscript®-T7T3 in vitro-transcription kit (Ambion/Applied Biosystems, Warrington, UK). The DNA was then destroyed with DNase I (2 units) and the mRNA precipitated and washed with 70% (v/v) ethanol. The mRNA sample was then reconstituted and the concentration and purity of RNA established. The identity of the TF mRNA was confirmed by end-point RT-PCR prior to use. One-step end-point RT-PCR was carried out using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech Inc, Giles, UK) and 100 ng of standard mRNA as before [25]. The amplification (30 cycles) was performed as follows: 1 min at 95°C, 1 min at 60 °C, followed by 1 min at 72°C. The primers used were (TF-forward) 5’-ACCTGGAGACAAACCTCGGAC-3’ and (TF-reverse) 5’-GAGTTTCTCCTTCAGCTCTGC-3’. Products were visualised on a 1.5% (w/v) agarose gel.

Sets of cells (2x10^5/ml) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h. Total RNA was isolated using the TRI-reagent system (Sigma) from 10^6 cells. Real-time RT-PCR was carried out using primer sets designed to detect TF and β-actin and optimised by adhering to criteria for successful analysis [26]. Single-step RT-PCR was carried out in triplicates using 100 ng of total RNA from each sample tested. A set of previously prepared standard TF mRNA ranging 0.05-10 ng was included. The reaction was carried out at an annealing temperature of 60°C using the PowerSYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Warrington, UK) on an iCycler thermal cycler (Bio-Rad, Hemel Hempstead, UK) and the data analysed. The primers used were:

TF-forward: 5’-TACAGACAGCCCGGTAGAGTG-3’,

TF-reverse: 5’-GAGTTTCTCCTTCAGCTCTGC-3’,

β-actin-forward: 5’-TGATGGTGCCATGGGTCAGA-3’,

β-actin-reverse: 5’-GTCGTCAGTTGCTGACGAT-3’
Following the reaction, the data were adjusted relative to the β-actin and then the exact TF mRNA quantities determined from the standard curve prepared using the *in vitro*-transcribed TF mRNA.

2.3. Measurement of total TF antigen, cell surface TF activity and released-TF activity

Sets of cells (2×10^5/ml) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h and in some instances, with 200 µg/ml for up to 6 days. To measure the total cellular TF antigen the cells were lysed in the presence of a protease inhibitor cocktail (Active Motif) and 20 µg of the samples analysed using a TF-antigen ELISA kit (Affinity Biologica, Ancaster, Canada) as described before [27]. Microparticle-derived TF was measured by ELISA directly. The TF concentrations were determined against a standard curve prepared simultaneously using recombinant TF (0-200 ng/ml) (American Diagnostica Inc., Stamford, USA). Additionally, the TF activities on the cells and released into the media were measured using a chromogenic assay based on quantifying the activity of the generated thrombin, as previously described [28].

2.4. Isolation of cell-derived microparticles and determination of microparticle density

Microparticles were isolated from conditioned media from each cell line by ultracentrifugation as described previously [29,30]. Briefly, the media were centrifuge in a microcentrifuge at 8,000 rpm for 10 min to remove cell debris. The microparticles were then sedimented by centrifuging at 100,000 g for 60 min at 20°C. The pellet was then resuspended in PBS and sedimented again and finally, the pellet was resuspended in 100 µl of PBS. Microparticle density was determined using the Zymuphen microparticle assay kit (Hyphen BioMed, Quadrathec, Epsom, UK) and equal amounts of microparticles were then used to measure the TF content as described above.
2.5. Cell transfection and quantification of NFκB activity and measurement of nuclear translocation of NFκB

Cells (5×10^5/well) pre-adapted to OptiMEM-1 media, were transfected with the Pathdetect pNFκB-Luc plasmid (1 µg, Stratagene, Cambridge, UK) using Lipofectin according to the manufacturer’s instructions. The cells were then incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h prior to measuring the luciferase activity as previously described [25,31]. Additionally, a set of untransfected cells was treated for 24 h with the NFκB inhibitor, pyrrolidinedithiocarbamate ammonium (Tocris Bioscience, Bristol, UK) (10 µM) prior to measuring TF antigen expression, as before [25]. In order to measure the nuclear localisation of NFκB, sets of cell lines were incubated for 24 h in the presence of LMWH (200 µg/ml) together with an untreated sample, a sample treated with pyrrolidinedithiocarbamate ammonium and a sample treated with betulinic acid (1.04 µM) to activate NFκB (30 min). Cellular nuclei were prepared from the samples using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium), and lysed in cell lysis buffer (Active Motif) containing protease inhibitors. The concentration of the total protein was determined using Bradford protein estimation reagent (Sigma). The samples were then diluted 1:1 (v/v) with 2× Laemmli’s sample buffer and similar quantities of protein were separated by 12% (w/v) SDS-PAGE electrophoresis. The proteins were then transferred onto nitrocellulose overnight, blocked in TBST buffer (Tris-HCl (20 mM) pH 8.0, 150 mM NaCl, Tween 20 (0.05% w/v)) and probed with a polyclonal anti-human NFκB-p65 antibody (eBioscience, Hatfield, UK) diluted 1:2000 (v/v) in TBST buffer, washed and probed with a goat anti-rabbit HRP-conjugated antibody (Santa Cruz Biotechnology) diluted 1:1000 (v/v) and developed using stabilised 3,3’,5,5 Tetramethylbenzidine (TMB)-stabilised substrate (Promega Corporation). Visible bands were recorded using the GeneSnap Program (SynGene). In addition, sets of
cells were treated as above but were fixed with 3 % (v/v) glutaraldehyde for 15 min and then washed with PBS. The cells were permeabilised using 0.2 % (v/v) Triton X-100 for 15 min and blocked with 10 % (w/v) goat serum (Santa Cruz Biotechnology) for 30 min. The rabbit anti-human p65 antibody was diluted 1:500 in PBS containing 1 % (w/v) BSA and cells incubated with the antibody solution for 1 h. The cells were then washed three times with PBS. A goat anti-rabbit FITC-conjugated antibody was diluted 1:500 in PBS containing 1 % (w/v) BSA and incubated with the cells for 1h. The cells were washed four times with PBS and the slide mounted in mounting media containing DAPI. Images were acquired using fluorescence imaging microscopy and captured using MetaVue software.

2.6. Influence of the supplementation of cells with EGF, bFGF or VEGF on the downregulation of TF by LMWH

Each cell line (2×10^5/ml of media) was supplemented with a range of concentrations of either EGF (0-10 ng/ml), bFGF (0-20 ng/ml) (Sigma Chemical Company Ltd.) or VEGF (0-4 ng/ml) (TCS Cellworks, Buckingham, UK) in the presence or absence of LMWH (200 µg/ml). The cells were incubated for 24 h and the amount of TF antigen determined by ELISA, as above.

2.7. Statistical analysis

Unless otherwise stated, all values presented are the mean value from the number of experiments stated, together with the derived standard error of 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.
3. Results

Prior to the study, the expression of TF in all five cell lines was confirmed and the cells were shown to exhibit a significant amount of TF mRNA, antigen and cell surface activity (data presented as untreated samples within the sections below). No significant change in level of apoptosis was detected in any of the LMWH-treated cells, compared to untreated cells.

3.1. Influence of LMWH on cellular TF expression and activity

At an optimal primer concentration of 100 nM, the real-time PCR efficiency was consistently greater than 83%. Furthermore, the amplification of the standard TF mRNA was linear over the range of 0.05-10 ng with a correlation coefficient of 0.989 and therefore minimum sensitivity of the reaction was assumed to be 0.05 ng of TF mRNA. The basal expression of TF mRNA was highest in LoVo cells (10.7 ± 3.0 ng/10⁶ cells), lower in BxPC-3 (7.9 ± 1.0 ng/10⁶ cells), MDA-MB-231 (8.6 ± 2.8 ng/10⁶ cells) and A375 cells (8.6 ± 0.3 ng/10⁶ cells) and lowest in SKOV-3 cells (7.7 ± 0.1 ng/10⁶ cells). Incubation of BxPC-3, LoVo and MDA-MB-231 cells for 24 h, with 20 µg/ml, or higher concentrations of LMWH resulted in the suppression of TF mRNA expression (Fig. 1A). In addition, the downregulation of TF mRNA expression was achievable with 200 µg/ml of LMWH in A375 and SKOV-3 cells. The expression of TF mRNA decreased at different rates in the five cell lines on incubation with LMWH (200 µg/ml) (Fig. 1B) but remained unaltered in untreated cells (not shown) adjusted to cell numbers.

Incubation of BxPC-3, LoVo and A375 cells with LMWH resulted in the reduction in detectable total TF antigen which was achievable at differing LMWH concentrations. However, the level of TF antigen did not drop below 60% of the untreated control in any of these cell lines (Fig. 2). In contrast, cellular TF decreased rapidly at high concentrations of
LMWH (200-2000 µg/ml) in MDA-MB-231 and SKOV-3 cells, diminishing to approximately 21% of the untreated control. Moreover, prolonged incubation of BxPC-3, LoVo and A375 cells with LMWH (200 µg/ml) suppressed the expression of TF protein over the 6 days (Fig. 2B) but remained unaltered in untreated cells (not shown) adjusted to cell numbers. In addition to the total TF antigen, incubation of all cells with LMWH resulted in the reduction in detectable cell surface TF activity, achievable at differing LMWH concentrations (Fig. 3A). The reduction in TF activity was also time-dependent (Fig. 3B) but remained unaltered in untreated cells (not shown). Pre-incubation of cells with a polyclonal TF-neutralising antibody (American Diagnostica Inc) resulted in no detectable chromogenic activity confirming the TF activity on the cells surface (not shown). Finally, the concentration of released TF into the conditioned media of the untreated samples remained unaltered on treatment with LMWH (0-2000 µg/ml) in all cell lines tested (Fig 4A). In contrast, continuous incubation of cells with LMWH (200 µg/ml) resulted in progressive decrease in microparticle-derived TF (Fig 4B) which approximated the pattern of surface-TF activity (Fig. 3B). The rate of release of microparticles was not significantly altered by any of the treatments and no additional TF antigen was detectable in microparticle-depleted conditioned media.

3.2. Influence of LMWH on NFκB transcriptional activity and nuclear translocation of NFκB

The Pathdetect-cis luciferase reporting system was optimised as previously described [25,31]. This is a quantitative technique that measures the transcriptional activity of NFκB taking into account influences from other signalling mechanisms which can interfere with the translocation of NFκB into the nucleus. Incubation of cells with a range of LMWH concentrations (0-2000 µg/ml) resulted in the dose-dependent suppression of basal NFκB activity in BxPC-3 and MDA-MB-231 cells (Fig. 5). LMWH was also effective in
suppression of basal NFκB activity in LoVo and SKOV-3, and in A375 cells at the higher concentrations (200-2000 µg/ml). Furthermore, incubation of all the tested cells with the NFκB inhibitor pyrrolidinedithiocarbamate ammonium (10 µM) resulted in decreases in TF antigen expression (Fig. 6A) that were comparable to inhibition levels achieved with LMWH (Fig. 2). Finally, inclusion of betulinic acid (1.04 µM) to activate NFκB, reversed the suppression of TF antigen expression by LMWH (200 µg/ml) (Fig. 6B). This data was consistent with the suppression of the nuclear translocation of NFκB upon treatment of cells with LMWH (200 µg/ml) which was comparable but lower than inhibition with pyrrolidinedithiocarbamate ammonium (Fig. 6C and Supplemental Fig. I show MDA-MB-231 cells). Furthermore, the level of nuclear NFκB in untreated cells was comparable to that induced by incubation with betulinic acid.

3.3. Influence of the supplementation of cells with EGF, bFGF or VEGF on the downregulation of TF expression by LMWH

Incubation of all cell lines with EGF (2.5-10 ng/ml) resulted in dose-dependent increases in TF expression, with the greatest increases observed in LoVo, SKOV-3 and A375 cells (supplemental Fig II). Furthermore, inclusion of LMWH (200 µg/ml) significantly reduced the TF expression in response to EGF in BxPC-3, MDA-MB-231 and SKOV-3 cells, but was partially counteracted on incubation with higher concentrations of EGF in LoVo, MDA-MB-231, SKOV-3 and A375 cells (Fig 7). Supplementation of cells with bFGF (5-20 ng/ml) also resulted in dissimilar increases in TF expression in BxPC-3, SKOV-3 and A375 cells (supplemental Fig II). Furthermore, inclusion of LMWH (200 µg/ml) resulted in the reduction in TF expression in all cell lines but was counteracted by increasing concentrations of bFGF in BxPc-3, MDA-MB-231 and SKOV-3 cells. Finally, incubation with VEGF (1-4 ng/ml) resulted in large increases in the expression of TF in SKOV-3 and A375 cells.
(supplemental Fig II), while the inclusion of LMWH (200 µg/ml) resulted in decreases in TF expression which were counteracted to differing extents at higher concentrations of VEGF in all cell lines (Fig. 7).

4. Discussion

It has previously been reported that treatment of patients with LMWH has beneficial influences beyond that of the immediate anticoagulant effect, through suppressing TF expression [17,18,32-35]. In these experiments, we explored the underlying mechanism by which LMWH suppresses TF expression in cancer cells. We have shown that incubation of cells with LMWH (20-2000 µg/ml) resulted in the suppression of TF mRNA expression, but became effective at various concentrations of LMWH in the different cell lines tested (Fig. 1). The suppression of TF mRNA expression was also time-dependent affecting the cells at different intervals. Downregulation of mRNA expression was concurrent with the suppression of the transcriptional activity and nuclear translocation of NFκB by LMWH at approximately the same effective concentrations in all cell lines tested (Fig. 5 and MDA-MB-231 cell shown in Fig. 6C and supplemental Fig. 1). However, NFκB was affected to a greater extent by LMWH in BxPC-3 and MDA-MB-231 cell lines than TF mRNA levels. It is known that in addition to the NFκB promoter region, the TF mRNA may also be transcribed through AP1, SP1 and EGR1 promoter sites [19]. Therefore, while the transcriptional activation of NFκB generates a large proportion of expressed TF mRNA in these cells, we cannot rule out additional contribution from the other promoters in these two cell lines. Consequently, although the NFκB activity is suppressed by incubation of cells with LMWH, the expression of TF mRNA is not attenuated proportionally due to the activity of other enhancer proteins. The function of NFκB in the regulation of TF expression in tall cells was confirmed by the inhibition of NFκB using an inhibitor while the involvement of NFκB in the mechanism of
inhibition by LMWH was demonstrated using the NFκB activator betulinic acid (Fig. 6). Although betulinic acids is also known to induce apoptosis in tumour cells, the concentrations used by us (1.04 µM) were far lower than those reported to induce apoptosis (15-80 µM) [36-38] and no cell apoptosis was detected in our experiments.

Decreases in both TF antigen and TF activity levels on incubation with LMWH were not to the same extent as the reductions in TF mRNA levels. Decreases in TF antigen levelled off at above 60% of the untreated control in BxPC-3, LoVo and A375 cells (Fig. 2) on incubation with high concentrations of LMWH (2000 µg/ml), suggesting the presence of a reservoir of intracellular TF that is not immediately depleted on suppression of TF mRNA expression. This is in agreement with the concept that TF exists in various pools within cells [39,40]. Moreover, the moderate decrease in total TF antigen in these cells was reflected in cell-surface TF activity which decreased to 72%, 55% and 65% of the untreated control in BxPC-3, LoVo and A375 cells respectively (Figs. 2A and 3A). In addition, the activity of TF released by these cells remained constant on incubation with LMWH (Fig 4A). In contrast, the level of TF antigen, and to a lesser extent TF activity, in MDA-MB-231 and SKOV-3 cells was reduced significantly on incubation with LMWH suggesting that the majority of the remaining cellular TF was present at the surface of these cells. Therefore, it may be hypothesised that MDA-MB-231 and SKOV-3 cells readily transfer and release TF into the media. In contrast, BxPC-3, LoVo and A375 cells may retain a higher proportion of TF within intracellular reservoirs. This mechanism explains the presence of high TF antigen levels in LoVo cells, despite the rapid decrease in TF mRNA. Furthermore, the rapid release of TF also explains the large reductions in TF antigen in SKOV-3 cells despite modest downregulation of TF mRNA, in response to LMWH. A previous study showed not effect of LMWH on tumour TF activity in mouse xenograft model of cancer [14]. However, in this model, LMWH was administered as a single bolus injection prior to the injection of the
tumour cells into the animals. In our study, the time-course of the downregulation of TF antigen and activity in BxPC-3, LoVo and A375 cell lines indicates that treatment of these cell lines with LMWH (200 µg/ml) resulted in the progressive downregulation of TF antigen expression (Fig. 2B) and release of TF (Fig 4B) over 6 days and was cumulative over the period of testing. The gradual reduction of cellular and released TF antigen further supports the presence of intracellular reservoirs of TF that replenish the surface TF. It is also noteworthy that the rate of microparticle release was independent of that of TF release and did not significantly alter during the period of testing. It is therefore possible that prolonged treatment with LMWH would be required for any effective long term control of TF-mediated hypercoagulable state. In this work, we were unable to sustain the cells for longer durations due to excessive cell proliferation. However, these data are in agreement with our previous clinical observations in pancreatic cancer patients indicating that prophylactic treatment with dalteparin may reduce the level of circulating TF antigen in the plasma of patients compared to those not receiving LMWH [18]. Consequently, our data indicate that therapeutic levels of LMWH, comparable to 20-200 µg/ml of the LMWH used here (activity = 0.1-1 units/ml) may be effective in reducing the risk of recurrent TF-induced thrombosis in a number of cancer cells. This study was carried out using LMWH obtained from Sigma Chemical Company Ltd, and confirmed using dalteparin. Therefore, one also has to take into consideration that different LMWH preparations used as pharmacological agents are likely to have varying anti-neoplastic properties [41] attributed to the varying length of fractionated heparin chains in these preparations, stemming from different methods of production. Therefore, clinical doses relevant for ‘secondary prophylaxis’ would have to be used in a twice daily schedule to achieve sustained levels of anticoagulant activity of 0.5-1.5 units/ml (3,500-10,500 units for a 70 kg average adult). In fact multiple daily doses of 5,000 units have been reported in the prophylactic treatment of cancer patients [42,43] and dalteparin
dosages of 2,500-18,000 units have been cited in protocols. These values are comparable to the concentrations used in this study (200 µg/ml = approximating 1 unit/ml) and therefore it is likely that attenuation of TF expression is realistic at these therapeutic doses.

In order to examine the hypothesis that LMWH downregulates TF expression through interfering with growth factor receptor signalling and subsequent suppression of basal NFκB transcriptional activity, we measured the effectiveness of LMWH (200 µg/ml) in suppressing TF expression in the presence of increasing concentrations of the growth factors EGF, bFGF and VEGF. The engagement of growth factor receptors with their receptors is known to induce NFκB activity [44] through parallel signalling pathways including the mitogen activated protein kinase pathway [45]. Furthermore, the increased expression of growth factor receptors is known to be an underlying cause of the hyperactivity of cancer cells to growth factors [46,47]. Our data suggest that LMWH may interfere with the interaction of the growth factors tested here (and possibly others) with their respective cell surface receptors. The interaction of heparin with bFGF and VEGF but not EGF is well documented. Therefore, one possible explanation for the observed decrease in the TF expression may involve interference with the action of heparin binding-EGF component co-purified with the isolated EGF preparation. In addition, increasing concentrations of growth factors partially reduced the effectiveness of LMWH in reducing TF expression (Fig. 7). Furthermore, the cell lines used in this study exhibited distinctive sensitivities to growth factors and therefore, were affected by LMWH to different extents. In particular, the expressions of TF in LoVo and A375 cells were neither significantly influenced on supplementation with the lower concentrations of LMWH (Fig 1A), nor were they greatly reversed by the inclusion of growth factors (Fig 7). This in turn may be indicative of a functional mutation in the growth receptors as discussed.
previously [48], rendering these hyper-responsive and therefore, less subject to normal cellular regulation.

5. Conclusions

The benefits of LMWH in the treatment of cancer patients have previously been reported [49]. Moreover, the beneficial effects of anticoagulants in cancer patients do not appear to be due to any direct antitumour effects and have been attributed to the suppression of the release of circulating TF [50]. Data obtained from this investigation suggest that LMWH is capable of downregulating the expression of TF mRNA through a mechanism which appears to involve the suppression of the transcriptional activity of NFκB and subsequently results in the gradual reduction of the intracellular TF reservoir. Since this stored TF can replenish the active cell-surface TF, the eventual depletion of the reserves would result in the lower levels of procoagulant cellular TF, and released TF-containing microparticles by the tumour cells. Therefore, the benefits of LMWH therapy may extend beyond the immediate inhibition of coagulation and sustained therapy may be advantageous in limiting the expression of TF.

Acknowledgement

The support of the Castle Hill Hospital Cancer Trust Fund is acknowledged.

References


Figure Legends

Figure 1 Analysis of the influence of LMWH on the expression of TF mRNA.

Sets of cells (2×10^5) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h (A) or with LMWH (200 µg/ml) for up to 6 days (B). Total RNA was isolated from the cells and analysed for TF expression by real-time quantitative RT-PCR. The data were normalised against β-actin mRNA in each sample and the quantity of RNA determined from a standard curve prepared by amplification of in vitro-transcribed TF mRNA. Data represent the mean of 3 experiments measured in triplicates (*=p<0.05 vs. respective untreated sample).

Figure 2 Analysis of the influence of LMWH on the expression of TF antigen.

Sets of cells (2×10^5) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h (A) or with LMWH (200 µg/ml) for up to 6 days (B). The cells were then lysed and analysed by ELISA and TF concentrations determined from a standard curve prepared with recombinant TF alongside. Data represent the average of 4 experiments measured in duplicates (*=p<0.05 vs. respective untreated sample).

Figure 3 Analysis of the influence of LMWH on TF activity.

Sets of cells (2×10^5) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h (A) or with LMWH (200 µg/ml) for up to 6 days (B). The cells were then resuspended in PBS and TF activity in samples (20 µl) measured using a chromogenic assay.
The absorption values were then converted into equivalent concentrations from a standard curve. Data represent the average of 3 experiments measured in duplicates (*=p<0.05 vs. respective untreated sample).

Figure 4 Analysis of influence of LMWH on released TF antigen

Sets of cells (2×10^5) were incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h (A) or with LMWH (200 µg/ml) for up to 6 days (B). The media from the cells was then collected and the microparticles isolated by ultracentrifugation. The microparticles density was then measured and the TF concentration in equal amounts of microparticles determined. Data represent the average of 4 experiments measured in duplicates (*=p<0.05 vs. respective untreated sample).

Figure 5 Analysis of the influence of LMWH on the transcriptional activity of NFκB.

Sets of cells were transfected with the pNFκB-Luc plasmid, incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h, lysed and luciferase activity measured. Data represent the average of 3 experiments measured in duplicates (*=p<0.05 vs. respective untreated sample).

Figure 6 The influence of NFκB activity on TF expression and suppression by LMWH and nuclear localisation of NFκB.

A) Cells (2×10^5) were treated for 24 h with the NFκB inhibitor, pyrrolidinedithiocarbamate ammonium (10 µM) alongside an untreated set and the concentration of TF antigen measured by ELISA. B) Cells (2×10^5) were treated for 24 h with LMWH (200 µg/ml) in the presence and absence of the NFκB activator, betulinic acid (1.04 µM) alongside an untreated set. The concentration of TF antigen was then measured by
ELISA. Data represent the average of 3 experiments measured in duplicates (*=p<0.05 vs. respective untreated sample; #=p<0.05 vs. respective sample treated with LMWH). C) Sets of cells were incubated for 24 h in the presence of LMWH (200 µg/ml) together with an untreated sample, a sample treated with pyrrolidinedithiocarbamate ammonium and a sample treated with betulinic acid (1.04 µM) to activate NFκB (30 min). Cellular nuclei were prepared from the samples using the Nuclear Extract Kit, lysed in cell lysis buffer containing protease inhibitors. The concentration of the total protein was then determined and diluted 1:1 (v/v) with Laemmli’s sample buffer and similar quantities of protein were separated by 12% (w/v) SDS-PAGE electrophoresis. Following transfer onto nitrocellulose, the membranes were blocked in TBST buffer and probed with a polyclonal anti human NFκB-p65 antibody diluted 1:2000 (v/v) in TBST buffer, washed and probed with a goat anti-rabbit HRP-conjugated antibody (1:1000 (v/v)) and developed using stabilised 3,3,'5,5-Tetramethylbenzidine (TMB)-stabilised substrate. All visible bands were recorded using the GeneSnap Program.

Figure 7 Analysis of the influence of EGF, bFGF and VEGF on the downregulation of TF by LMWH.

Sets of cells (2×10^5) were incubated with a range of concentrations of EGF (0-10 ng/ml), bFGF (0-20 ng/ml) or VEGF (0-4 ng/ml) in the presence or absence of LMWH (200 µg/ml) for 24 h. The cells were then lysed and analysed by ELISA and TF concentrations determined from a standard curve prepared alongside. Percentage reduction in TF antigen was determined against samples containing the same concentrations of growth factor but devoid of LMWH. Data represent the average of 3 experiments measured in duplicates. *=p<0.05 for the alteration in the level of TF suppression against the respective sample, without growth factor.