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

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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 whilst 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.

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

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

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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 colocarcinoma 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 were confirmed as described below. Cells were supplemented with a range of LMWH concentrations (Mr ~3000) (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′-GAGTTCTCCTTCCAGCTCTGC-3′. Products were visualised on a 1.5% (w/v) agarose gel.

Sets of cells (2×10^5/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′-GAGTTCTCCTTCCAGCTCTGC-3′,
β-actin-forward 5′-TGATGGTGGGCATGGGTCAGA-3′,
β-actin-reverse 5′-GTCGTCCCAGTTGGTGACGAT-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 Biologicals, 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 serum-free conditioned media from each cell line by ultracentrifugation as described previously [29,30]. Briefly, the media were centrifuge in a microcentrifuge at 8000 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, Quadratech, 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)