Antimetastatic effect of tinzaparin, a low-molecular-weight heparin

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Summary. The importance of coagulation activation in cancer patients is suggested by the clinical finding of hypercoagulability, experimental enhancement of metastasis and angiogenesis by coagulation factors such as tissue factor (TF) and thrombin and the possible antitumor effects of anticoagulant agents. Tinzaparin is a low-molecular-weight heparin (LMWH) with a relatively high molecular weight distribution and high sulfate to carboxylate ratio. In addition to its ability to inhibit thrombin and factor Xa, tinzaparin is particularly effective at releasing endothelial tissue factor pathway inhibitor (TFPI), the natural inhibitor of both procoagulant and non-coagulant effects of TF. The present study was undertaken to investigate the effects of tinzaparin on lung metastasis using a B16 melanoma model in experimental mice. Tinzaparin's anticoagulant effect in mice and its ability to release TFPI from human endothelial cells at various time points were demonstrated. Subcutaneous (s.c.) injection of tinzaparin (10 mg kg⁻¹) 4 h before intravenous administration of melanoma cells (2.0 × 10⁵) markedly (89%) reduced lung tumor formation (3 ± 2) compared with controls (31 ± 23; P < 0.001). In a second group of animals, tinzaparin (10 mg kg⁻¹, s.c.) administered daily for 14 days following the initial (pretumor cell) dose, before assessment of lung seeding, reduced tumor formation by 96% (P < 0.001). No bleeding problems were observed in any of the tinzaparin-treated animals, despite a 4-fold prolongation of the whole blood clotting time after a single s.c. dose of tinzaparin (10 mg kg⁻¹). Administration of tumor cells (2 × 10⁵) caused a rapid and significant fall in platelet count 15 min after injection (a sensitive marker of intravascular coagulation) in controls (939 ± 37 vs. 498 ± 94 × 10⁹ mL⁻¹, P < 0.01), but this was prevented by tinzaparin treatment (921 ± 104 × 10⁹ mL⁻¹). These data provide further experimental evidence to support the potential for LMWH as antimetastatic agents.

Keywords: low-molecular-weight heparin, metastasis, thrombosis, tissue factor pathway inhibitor.

Introduction

The link between coagulation and malignancy has been recognized for over a century and is now well accepted [1,2]. Hypercoagulability in cancer patients is extremely common and is usually associated with a chronic low-grade intravascular coagulation [2,3]. Thrombosis is a common cause of death in cancer patients and pulmonary embolism (PE) is more commonly present in cancer patients studied at autopsy than in those without malignancy [4]. It may also be the presenting symptom of an underlying malignancy [5].

It is now evident that coagulation and platelet activation in malignancy may also have significance in the biology of tumor growth and dissemination. Thus, there is mounting evidence that components of tumor-induced hypercoagulability may not only lead to thrombosis, but may also enhance tumor angiogenesis and metastasis [6–8]. A number of factors that positively or negatively regulate angiogenesis are related to the coagulation pathway [9,10]. These include platelet-derived products such as vascular endothelial growth factor (VEGF), platelet factor 4, and thrombospondin, and terminal products of coagulation including fibrin and its fibrin degradation products. There has also been considerable research interest in a possible role of tissue factor (TF) in tumor biology. This protein, which normally acts as a cellular receptor for factor (F)VIIa and initiates the coagulation pathway [11], is present on the surface of many tumor cell types and is largely responsible for tumor cell procoagulant activity (PCA) [12]. Tumor cell-induced thrombin generation via TF/FVIIa and subsequent platelet activation has been shown to enhance hematogenous metastasis in ex-

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Experimental animal models [8,12]. TF has also been shown to promote metastasis via non-procoagulant functions [13].

The effects of unfractionated heparin have been studied both as a single agent and in combination with other drugs in animal models of malignancy. While some studies showed that heparin delayed the growth of implanted primary tumors [14], most demonstrated an inhibitory effect against metastasis [15–18]. Furthermore, co-operative effects of heparin with other molecules such as α2-glycoprotein, interferon and tumor necrosis factor against tumor growth and metastasis have been reported [19,20]. We previously demonstrated that the significant fall in peripheral blood platelet count that follows intravenous (i.v. tail vein) injection of procoagulant fibrosarcoma cells in experimental animals corresponds to the accumulation of platelets in the lungs (the organ of first encounter) [8]. Both tumor cell-induced thrombocytopenia and experimental metastasis can be significantly inhibited by warfarin as well as unfractionated heparin [8,21].

Tinzaparin (Innohepᵀᴹ; Pharmion Corporation, Boulder, CO, USA) is the most recent low-molecular-weight heparin (LMWH) to be approved by the US Food and Drug Administration. It is produced by controlled heparinase depolymerization of unfractionated porcine heparin and has an average molecular weight of 6500 Da (Fig. 1). Tinzaparin is indicated for the treatment of acute symptomatic deep vein thrombosis (DVT) with or without PE when administered in conjunction with warfarin [22]. Typically, tinzaparin is given subcutaneously once daily without the need for laboratory monitoring [23]. Similar to the other LMWHs, tinzaparin inhibits factors (F)Xa and (F)IIa by binding to the plasma protease inhibitor antithrombin. Furthermore, tinzaparin is particularly effective at releasing tissue factor inhibitor (TFPI) from endothelial cells [24] and we have previously shown that TFPI exerts significant antitumor effects in experimental models of hematogenous metastasis [25]. The aim of the present study therefore was to determine the effect of subcutaneous (s.c.) tinzaparin on tumor cell-induced thrombocytopenia and experimental metastasis.

**Materials and methods**

Tinzaparin is made by the controlled enzymatic depolymerization of heparin isolated from porcine intestinal mucosa using heparinase from Flavobacterium heparinum. A 2-O-sulfate-4-enepyranoicosuronic acid comprises the majority of the components at the non-reducing end and a 2-N, 6-O-disulfato-D-glucosamine structure at the reducing end of their chain (Fig. 1).

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (Walkersville, MD, USA). HUVEC were grown to 80–90% confluence in endothelial growth medium (EGM) containing hEGF (10 ng mL⁻¹), hydrocortisone (1 mg mL⁻¹), gentamicin (50 μg mL⁻¹), amphotericin-B (50 μg mL⁻¹), blood brain extract (0.012 mg mL⁻¹) and 2% fetal bovine serum (FBS) equilibrated with 95% air/5% CO₂ at 37°C. HUVEC cells were serially passaged and maintained in endothelial growth medium in cell culture flasks coated with 0.2% gelatin (Sigma Chemical Co., St Louis, MO, USA). Confluent cultures of endothelial cells between the third and sixth passages were washed with Hank’s balanced salt solution and harvested with a solution containing 0.025% trypsin and 0.01% EDTA and counted.

**Release of endothelial TFPI**

The effect of tinzaparin (1 μg) on endothelial cell TFPI release into culture media over time was examined. TFPI was measured in culture medium supernatants by enzyme-linked immunoassay following incubation in microtiter wells precoated with a rabbit antihuman TFPI polyclonal antibody. Bound TFPI was detected using a biotinylated monoclonal antibody specific for the Kunitz domain 1 of TFPI [26]. Subsequent binding of the streptavidin-conjugated horseradish peroxidase (HRP) was detected colorimetrically after addition of tetramethylbenzidine substrate. TFPI levels were calculated from a standard calibration curve generated using purified TFPI.

**Tumor cells**

B16 murine malignant melanoma cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, penicillin and streptomycin (Sigma). They were cultured to 70% confluence and harvested with trypsin-EDTA (Sigma) and washed twice with PBS. Cells were resuspended in PBS at a concentration of either 1 × 10⁶ cells mL⁻¹ for experimental metastasis or at 1 × 10⁷ cells mL⁻¹ for tumor cell-induced thrombocytopenia experiments.

**Animals**

Female C57/B16 mice (Harlan, Indianapolis, IN, USA) weighing 18–21 g were used for this study. All procedures were in

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acquaintance with Institutional Animal Care and Use Committee and institutional guidelines.

**Anticoagulant effect of tinzaparin**

The anticoagulant effect of tinzaparin was measured 4h after s.c. or 15 min after i.v. (tail vein) injection of tinzaparin (10 mg kg⁻¹) in experimental mice. Blood samples were collected by cardiac puncture (0.5–1.0 mL) into plastic tubes containing 3.2% citrate (9:1 v/v). Platelet counts were determined electronically using a Coulter MD electronic counter (Coulter Electronics, Miami, FL, USA). Whole blood clotting times were measured in a Sonoclot™ Coagulation Analyzer after recalcifying whole blood (400 µL) with 0.1 M CaCl₂ (40 µL). Activated partial thromboplastin times (APTT) were measured on citrated plasma using the Amax coagulation analyzer (Sigma).

**Effect of tinzaparin on tumor cell-induced thrombocytopenia**

Tinzaparin (10 mg kg⁻¹) was administered subcutaneously as described above. This relatively high level of tinzaparin was chosen to ensure adequate anticoagulation according to our observations that clotting occurs faster in mice than in humans, as well as considering the fact that coagulation factors and platelet numbers are higher in mice compared with humans [27].

Control animals were injected subcutaneously with PBS. Tumor cells (2 × 10⁶) were injected intravenously via the tail vein 4 h after tinzaparin administration. Platelet counts were measured in both control and tinzaparin-treated animals, before and 15 min after i.v. injection of tumor cells.

**Effect of tinzaparin on experimental metastasis**

Tinzaparin was administered by s.c. injection (10 mg kg⁻¹ in 0.2 mL), followed 4 h later by the i.v. injection of tumor cells (2.0 × 10⁵ in 0.2 mL PBS) via the tail vein. In a second experimental group, in addition to the pretumor cell dose, tinzaparin (10 mg kg⁻¹) was also administered subcutaneously daily for 14 days. Control animals received PBS. No bleeding problems were observed in any of the tinzaparin-treated animals. On day 15, animals were killed by an overdose of Halothane anesthetic and the lungs were removed from the thoracic cage en bloc, washed in PBS and fixed in Bouin’s solution. Pulmonary tumor nodules on the surface of the lungs were counted macroscopically.

**Statistical analysis**

All data were normally distributed as assessed by normal probability plots (Statistica™ for Windows). Summary statistics were therefore presented as means and standard deviation and differences between groups were analyzed using Student’s t-test. Throughout the work statistical significance was assumed when P < 0.05.

**Table 1** Effect of tinzaparin (1 µg) on the release of tissue factor pathway inhibitor (TFPI) from human endothelial cells (HUVEC)

<table>
<thead>
<tr>
<th>Time (h) post tinzaparin</th>
<th>Mean (± SD) TFPI (ng/2 × 10⁶ cells mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>12.5 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>38.0 ± 3.5</td>
</tr>
<tr>
<td>8</td>
<td>52.5 ± 5.4</td>
</tr>
</tbody>
</table>

**Results**

**Anticoagulant and TFPI-releasing effects of tinzaparin**

Four hours after s.c. injection of tinzaparin (10 mg kg⁻¹), the whole blood recalcification time was prolonged 4-fold (P < 0.001). APTT results were >200 s, also reflecting the presence of a significant circulating anticoagulant effect in the tinzaparin-treated animals. No changes in platelet count were observed in tinzaparin-treated animals compared with control (data not shown). At the dose employed (10 mg kg⁻¹) tinzaparin was well tolerated and no bleeding or any other apparent side-effects were observed during the study. The ability of tinzaparin to release TFPI from endothelial cells was demonstrated in vitro. Treatment of HUVECs with tinzaparin (1 µg) resulted in a marked and time-dependent release of TFPI, rising to >50 ng/2 × 10⁶ cells mL⁻¹ after 8 h (Table 1).

**The effect of tinzaparin on tumor cell-induced thrombocytopenia**

In control animals, the peripheral blood platelet count (initially 936 ± 37 × 10⁵) fell rapidly (498 ± 94 × 10⁵) following i.v. injection of tumor cells (P < 0.01; Fig. 2). In tinzaparin-treated animals, in contrast, no such effect on platelet count was observed (921 ± 104 × 10⁵) following tumor cell injection. Thus, pretreatment with tinzaparin effectively abolished tumor cell-induced thrombocytopenia.

![Platelet Count](image)

**Fig. 2.** The effect of tinzaparin on tumor cell-induced thrombocytopenia. Melanoma cells were injected 4h after subcutaneous administration (10 mg kg⁻¹) of tinzaparin. Blood (n = 4) was collected for platelet count 15-30 min after tumor cell injection. Tinzaparin completely abolished the thrombocytopenia caused by intravenous tumor cell injection.
Activation of coagulation, with subsequent generation of thrombin, is believed to occur primarily as a result of TF expression by the tumor cells. We have previously shown that inhibiting TF activity with a monoclonal antibody effectively prevented melanoma-induced thrombocytopenia and subsequent lung seeding [34]. Also, we have specifically demonstrated (by flow cytometry) that TF is present on the surface of B16 melanoma cells [25]. The thrombocytopenia that is observed following i.v. tumor cell injection is secondary to thrombin generation since B16 cells did not cause any significant activation of platelets directly as determined by agglutination using washed platelets, platelet-rich plasma or whole blood (data not shown). In addition, the observations that anti-TF antibodies, TFPI and heparins all inhibit tumor cell-induced platelet aggregation in vivo provide strong evidence to support the role of thrombin in that process.

Expression of αvβ3 on cancer cells and platelets is stimulated by thrombin which is generated by tumor cell procoagulant activity or due to vascular damage. Thrombin generation can promote tumor cell adhesion to the vascular endothelium [35–37]. The tumor cell-induced thrombin production and subsequent platelet aggregation are positively correlated with cancer progression and metastatic potential [38–40]. Thus it is likely that in the injectable model of metastasis used in the present study, tinzaparin is interfering with the initial adhesion and arrest of blood-borne melanoma cells by inactivation of thrombin and inhibition of platelet aggregation. Heparin treatment has also been shown to reduce the formation of ‘spontaneous’ lung metastasis in mice from subcutaneously implanted mammary carcinoma and improve survival of the animals [41].

Using several different approaches, we also demonstrated that TFPI is effective in this experimental model. First we showed that injection (i.v.) of recombinant TFPI significantly reduced the metastasis of B16 melanoma. Second, we found that stably transfected TFPI (+) B16 melanoma cells produced significantly fewer lung metastases compared with wild type and vector control cells. Finally, we demonstrated that mice receiving i.v. somatic gene transfer of TFPI expression vector developed significantly fewer lung nodules than controls [25]. Heparin is capable of releasing TFPI from vascular endothelial cells [42]. Among the LMWHs, tinzaparin is particularly effective at releasing TFPI [43] and this may therefore represent an additional mechanism by which this LMWH exerts an antineoplastic effect. In the present study, we confirmed the ability of tinzaparin to release endothelial TFPI, showing that release from HUVECs was time-dependent and continued for at least 8 h after tinzaparin exposure. This is broadly compatible with the observation that tinzaparin [single s.c. dose of 1751U kg⁻¹ (DVT treatment dose)] to healthy volunteers rapidly causes an increase in plasma TFPI, which is sustained for up to 2–5 h after injection, and only returns to basal levels after 16 h [43,44]. Of potential importance in cancer therapy is the observation that tinzaparin and its relatively higher molecular weight and sulfated fractions are more potent stimulants of endothelial TFPI release compared with other LMWHs [43].
In summary, s.c. administration of tinzaparin effectively prevents tumor-associated coagulopathy and lung seeding in the B16 melanoma model of experimental metastasis. The favorable pharmacokinetic attributes of this agent compared with unfractionated heparin, together with its superior ability to release TFPI for relatively long periods from vascular endothelial cells compared with other LMWHs, provide a rationale for its use in oncology as an antimitotic as well as an antangiogenic agent.

References
42. Allian S, Gastpar P. Plasma levels of total and free tissue factor pathway inhibitor (TFPI) as individual pharmacological parameters of various heparins. Thromb Haemost 2001; 85: 824–9.