Abstract. Inhibitors of angiogenesis are potential anti-cancer agents in that they deprive tumors of the blood necessary for growth and metastasis. The anti-angiogenic efficacy of tinzaparin, a known anticoagulant low molecular weight heparin (LMWH), was examined in vitro in endothelial cell tube formation assay and in vivo in the chick chorioallantoic membrane model. The observed anti-angiogenic effects of tinzaparin were shown to be dose-related and dependent on the relatively higher molecular weight tinzaparin fragments. These experiments demonstrated that tinzaparin is a potent inhibitor of angiogenesis (ED₉₀-₁₀₀ range, 0.05-0.1 mg) regardless of the angiogenic factor and suggest that its effect is mediated via cellular release of tissue factor pathway inhibitor (TFPI). This was evident by the reversal of either tinzaparin or r-TFPI anti-angiogenesis efficacy by a specific monoclonal TFPI antibody. The ED₉₀-₁₀₀ for the inhibition of angiogenesis for r-TFPI ranged from 0.01 to 0.03 mg in the chorioallantoic membrane model regardless of the pro-angiogenic factor. In addition, either tinzaparin or r-TFPI inhibited the growth of colon carcinoma tumors, human fibrosarcoma tumors, and human lung carcinoma in the chorioallantoic membrane tumor implant model. Thus, the LMWH tinzaparin, in addition to its anticoagulant effects, may offer important benefits in treatment of cancer and other disorders supported by pathologic angiogenesis.

Introduction

Angiogenesis is an essential feature of normal biologic processes, such as growth, development, reproduction, and repair of damaged tissue (1-3). Endogenous promoters and inhibitors regulate the complex process of angiogenesis (2). The later stages involve proliferation and organization of endothelial cells (ECs) into tube-like structures. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are important promoters of this process; these promoters exert their effects by binding to cell surface receptors. Human ECs in culture can form tube-like structures with lumens, and they represent an in vitro model system for the study of the angiogenesis process. Pathologic angiogenesis, which may occur when a normal control mechanism is defective, contributes to the growth and metastasis of tumors (3), as well as to inflammatory and certain ocular diseases.

Drugs that inhibit angiogenesis may be effective in the treatment of these human disorders (3). The mechanisms by which anti-angiogenic drugs exert their effects can vary widely, acting on different points in the complex process of tumor angiogenesis. Potential points of control include blocking the action of endogenous stimulators; inhibiting the growth, migration, and tube formation of ECs; and inhibiting the turnover of the capillary basement membrane (1). The role of heparin in angiogenesis modulation and its potential anti-cancer effect has been previously described, but without clear delineation of its pro- versus anti-angiogenic effect, as well as its mechanism of action (4,5).

Tinzaparin, a low molecular weight heparin (LMWH), has an average molecular weight of 6.5 kDa; it is produced by enzymatic degradation of heparin and has proven efficacy in the treatment of deep vein thrombosis and pulmonary embolism (6,7). Tinzaparin's antithrombotic activity is mediated by binding to microvascular tissues and activation of antithrombin III, a potent anticoagulant (8). Tinzaparin also causes release of tissue factor pathway inhibitor (TFPI), an important endogenous inhibitor of TF/VIIa (9). Several clinical trials have shown improved survival of cancer patients following heparin therapy (10-12). In one double-blind, multicenter clinical trial, tinzaparin was shown to be effective in the treatment of proximal deep vein thrombosis in a patient population that included a large percentage of cancer patients (6). These clinical data suggest that tinzaparin may have some benefit in the treatment of cancer patients. The present study was undertaken to elucidate the mechanisms through which tinzaparin may affect tumor angiogenesis and to assess its efficacy in inhibiting the angiogenesis processes.

Materials and methods

Endothelial tube formation assay. Human umbilical vein ECs (HUVECs) were obtained from Clonetics (Walkersville, MD, USA). Cells were grown to 80-90% confluence in endothelial growth medium (EGM) containing recombinant human endo-
thelial growth factor (hEGF; 10 ng/ml), hydrocortisone (1 mg/ml), gentamicin (50 mg/ml), amphoterin-B (50 µg/ml), blood brain extract (0.012 mg/ml), and 2% fetal bovine serum equilibrated with 95% air and 5% CO₂ at 37°C (13).

The basic form of FGF-2 was purchased from Gibco BRL (Gaithersburg, MD, USA) and R&D Systems (Minneapolis, MN, USA). Cells were serially passed in cell culture flasks coated with 0.2% gelatin (Sigma, St. Louis, MO, USA), and confluent cultures between the third and sixth passages were washed with Hank's balanced salt solution, harvested with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid, and counted by hemocytometer. HUVECs were re-suspended in 24-well plates coated with Matrigel matrices or directly on 96-well cell culture plates. Matrigel growth factor reduced medium was removed, and cells were fixed and stained using a modified Hema 3 Stain kit (Fisher, Swedesboro, NJ, USA).

Chorioallantoic membrane angiogenesis model. Ten-day-old embryos were purchased from Spafas, Inc. (Preston, CT, USA) and incubated at 37°C with 55% humidity. A small hole was punctured in the shell concealing the air sac; a second hole was punctured in the shell on the broad side of the egg was punctured in the shell on the broad side of the egg. A window, approximately to the first hole, which caused the chorioallantoic membrane beneath the second hole by the application of negative pressure as observed during candling (14). A false air sac was created directly over the avascular portion of the embryonic membrane, and the number of blood vessels entering the tumors was counted (as vessel branch points). Each treatment group incorporated at least ten tumors per experiment. Tumors were then excised from the egg, and tumor weights were determined for each CAM. Data are presented as mean tumor weight per treatment group ± SEM. Statistical analysis was performed using the Student t-test, p<0.05.

Results

LMWH and TFPI inhibit EC tube formation. A pivotal stage of angiogenesis is the formation of tube-like structures from ECs (1), a process that is mediated by cytokines binding to EC surface receptors (15). In this investigation we used an in vitro assay with cultured HUVECs capable of forming tube-like structures (13). The results show that FGF-2 (16) stimulates EC tube cell formation. Addition of tinzaparin reverses the FGF-2 effect in a dose-related fashion; tinzaparin (0.5 µg) completely reverses the FGF-2 effect to about the same level as seen in the PBS control (Table I). The effect of r-TFPI was also examined using the same assay. Like tinzaparin, r-TFPI at 12.5 ng significantly inhibited formation of EC tube formation induced by FGF-2. In contrast, a potent and specific anti-Xa (r-TAP) resulted in marginal effect on FGF-2-induced EC tube formation as compared with tinzaparin (Table I).

Microscopic analysis of CAM sections. CAM tissue directly beneath the FGF-2-saturated filter disk was resected from embryos treated 48 h previously with compounds or controls. Tissues were washed three times with PBS. Sections were placed in a 35-mm Petri dish obtained from Nalge Nunc (Rochester, NY, USA) and examined under an SV6 stereo-microscope from Karl Zeiss (Thornwood, NY, USA) at x50 magnification. Digital images of CAM sections adjacent to filters were collected using a 3-CCD color video camera system from Toshiba America (New York, NY, USA) and were analyzed with the Image-Pro Plus software from Media Cybernetics (Silver Spring, MD, USA). The number of vessel branch points contained in a circular region equal to the area of a filter disk was counted for each section (14). Percent inhibition data are expressed as the quotient of the experimental value minus the negative control value, divided by the difference between the positive control value and the negative control value.

Endothelial TFPI release assay. HUVEC TFPI is synthesized and constitutively secreted by ECs, and a major portion of intravascular TFPI is stored in association with ECs. TFPI has valuable effects on various cellular and vascular events that might broaden the efficacy and utility of heparin and derivatives. The effect of heparin molecular weight fractions ranging from 1,700 to 12,000 kDa and different LMWHs on the release of TFPI from HUVECs was determined.

CAM tumor implant model. Ten million tumor cells were placed on the surface of each CAM (7-day-old embryo) and were cultured for 1 week. The resulting tumors were excised and cut into 50-mg fragments. These fragments were placed on additional CAMs and treated topically the following day with tinzaparin, r-TFPI, or PBS (Fig. 2). Seven days later, CAMs were excised from the egg, and the number of blood vessels entering the tumors was counted (as vessel branch points). Each treatment group incorporated at least ten tumors per experiment. Tumors were then excised from the egg, and tumor weights were determined for each CAM. Data are presented as mean tumor weight per treatment group ± SEM. Statistical analysis was performed using the Student t-test, p<0.05.

Reversal of the inhibitory efficacy of tinzaparin on EC tube formation by anti-TFPI. The inhibitory efficacy of tinzaparin or r-TFPI on FGF2-induced EC tube formation was totally reversed by a specific monoclonal TFPI antibody (Table I). Similar data were shown for this antibody in reversing the inhibitory effect of tinzaparin or r-TFPI on TF/VIIa-induced EC tube formation (data not shown).
Figure 1. Diagrammatic sketch of the protocol used for the chorioallantoic membrane model (CAM) (in ovo).

Figure 2. Diagrammatic sketch of the protocol used for the 7-day chick embryo tumor growth model.
LMWH and TFPI inhibit angiogenesis in the CAM model. The anti-angiogenic effect of tinzaparin was further investigated utilizing a chick CAM assay (14). In this assay, the number of new blood vessel branch points that form following treatment provides a size-independent measure of the sprouting of new vessels that occurs during angiogenesis. Treatment with FGF-2 promoted formation of new blood vessel branch points by 2- to 3-fold above baseline. However, CAM tissue treated with FGF-2 and tinzaparin exhibited reversal of FGF-2-induced angiogenesis (Fig. 3). This anti-angiogenic reversal was dose-dependent (Fig. 3). Similar results were shown with r-TFPI in inhibiting FGF-2-induced angiogenesis. Treatment of CAM tissue with lipopolysaccharide, another angiogenesis promoter (12), also promoted formation of new blood vessel branch points. The reversal of lipopolysaccharide-induced angiogenesis was also observed with r-TFPI or tinzaparin (Table II). Additionally, angiogenesis mediated by other angiogenic factors, such as VEGF or tissue factor, was inhibited by tinzaparin (Table II). Similar anti-angiogenesis effects were demonstrated with r-TFPI in inhibiting VEGF and tissue factor-induced angiogenesis (Table II).

LMWH-induced TFPI release from human ECs. In an effort to further characterize and evaluate tinzaparin’s effect on angiogenesis and TFPI release, different molecular weight fractions of tinzaparin (ranging from 1,700 to 12,623 Da) were obtained by gel filtration. These various molecular weight fragments were evaluated for their ability to release TFPI from ECs and their ability to inhibit angiogenesis. Results from these experiments revealed that relatively higher molecular weight tinzaparin fragments were more effective in releasing TFPI from ECs in a concentration-dependent fashion and in inhibiting FGF-2-induced angiogenesis in the CAM model (Table III).

Table I. Inhibition of FGF-2-induced EC tube formation by LMWH but not by anti-Xa.\(^a\)

<table>
<thead>
<tr>
<th>Agents tested</th>
<th>Mean % inhibition of EC tube formation induced by FGF-2 ± SD</th>
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<tbody>
<tr>
<td>Tinzaparin (3 µg)</td>
<td>100±0</td>
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<tr>
<td>r-TAP (5 µg)</td>
<td>15±5</td>
</tr>
<tr>
<td>r-TFPI (0.5 µg)</td>
<td>98±6</td>
</tr>
<tr>
<td>r-TFPI (0.5 µg) + anti-TFPI (5 µg)</td>
<td>5±3</td>
</tr>
<tr>
<td>Tinzaparin (3 µg) + anti-TFPI (5 µg)</td>
<td>12±4</td>
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\(^a\)Mediation by TFPI. EC, endothelial cell; FGF-2, fibroblast growth factor-2; LMWH, low molecular weight heparin; TAP, tick anticoagulant peptide; TFPI, tissue factor pathway inhibitor.

Figure 3. Effect of tinzaparin on fibroblast growth factor-2 (FGF-2) promoted new blood vessel formation in the chorioallantoic membrane (CAM) model. Representative illustration showing the effect of FGF-2 and FGF-2 plus tinzaparin at different doses (A, 0.1 mg; B, 0.01 mg; C, 0.001 mg) on new blood vessel formation. At least ten CAMs were used per treatment group, and the experiment was repeated three times.
In reality, LMWH fractions in the range of hexasaccharide to octasaccharide molecular weight, Da, released angiogenesis inhibitor TFPI (Mean TFPI release). In the present experiments, the higher molecular weight fractions ranging from 6 to 8 kDa. These data further support the specificity of tinzaparin’s anti-angiogenic properties. Angiogenesis is a highly regulated process that is critical for development and wound healing in the adult organism.

**Discussion**

Angiogenesis is a highly regulated process that is critical for development and wound healing in the adult organism. Although placid for the most part, when angiogenesis becomes active, it can support the induction of a variety of human disorders, including cancer, inflammatory disease, and ocular disease. Thus, the discovery of pharmacological agents that possess anti-angiogenic properties could be therapeutically beneficial. The results of these experiments indicate that tinzaparin, an LMWH used to treat venous thrombosis, has significant potential as an anti-angiogenic drug. The potent anti-angiogenic effect of tinzaparin observed in the present experiments appears to be mediated by induction of TFPI release, an endogenous inhibitor of angiogenesis, since tinzaparin’s anti-angiogenic activity in a variety of *in vitro* and *in vivo* models appears to be synchronized with its ability to release TFPI.
These experiments support the view that LMWHs have the potential to be used in the treatment of human cancers and other disorders supported by pathological angiogenesis.

References