Heparins, cancer and thrombosis: clinical and experimental studies
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TREATMENT WITH UNFRACTIONATED HEPARIN OR LOW MOLECULAR WEIGHT HEPARIN DOES NOT AFFECT GROWTH OF SARCOMAS AND TUMOR-ASSOCIATED ANGIOGENESIS IN AN EXPERIMENTAL RAT MODEL

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ABSTRACT
A significant proportion of cancer patients receive anticoagulants, including heparins, to treat or prevent thromboembolism. Recent randomized trials that compared low molecular weight heparin (LMWH) to unfractionated heparin (UFH) for the initial treatment of deep vein thrombosis have indicated differential effects of these heparins on survival of patients with cancer, in favor of LMWH. Experimental studies have shown that UFH has activities besides its anticoagulant function which may affect progression of malignancy, including stimulation of new blood vessel formation. In contrast, it has been suggested that LMWH can inhibit angiogenesis. In the present study, we compared quantitatively the effects of in vivo treatment with UFH, LMWH, or placebo on growth of highly vascularized osteosarcomas and formation of blood vessels in these tumors in an experimental rat model. The results reveal that UFH and LMWH in therapeutic dosages do not affect growth of sarcomas or tumor blood vessel formation in vivo. Because of the wide variety of activities of heparins, it is concluded that the ultimate effect of heparin treatment on cancer progression is uncertain and appears not to be at the level of angiogenesis.

INTRODUCTION
Cancer patients have an increased risk of venous thromboembolic complications. Consequently, a significant proportion of cancer patients receive anticoagulants to treat or prevent thrombosis. Intravenous dose-adjusted unfractionated heparin (UFH) has been the standard initial treatment for venous thromboembolism for several decades, but recent randomized trials have shown that low molecular weight heparin (LMWH) is as safe and effective as UFH, and easier to administer (4). Interestingly, these trials have also indicated that either UFH or LMWH, or both anticoagulants, affect survival of patients with malignancy (17). Cancer patients treated with LMWH for their venous thromboembolism showed a significantly improved 3 month survival as compared to UFH recipients with cancer, whereas the incidences of thromboembolic recurrences and hemorrhages were identical in both treatment groups (17).
Experimental studies support the hypothesis that UFH and other glycosaminoglycans can affect cancer progression (11;36). Various steps in tumor growth or metastasis can be affected by UFH, and these effects are not solely related with its anticoagulant function. UFH has been found to affect the immune system (41), proliferation (3), migration (1;25) and invasion (5;18) of cancer cells and angiogenesis (24;28). The effects of LMWH on cancer are less thoroughly investigated. Interestingly, in vitro and in vivo studies have shown that UFH and LMWH differentially affect angiogenesis (6;24;28). UFH can stimulate angiogenesis by liberation of angiogenic growth factors from the extracellular matrix and enhancement of their activity by facilitating binding to their high affinity receptors (12;31;40;42). In contrast, LMWH can inhibit activity of angiogenic growth factors in vitro (19;38) and suppress angiogenesis in vivo (24;28). Recent in vitro experiments have indicated that heparins can also affect angiogenesis by altering the structure of fibrin matrices (6).
When UFH is present during polymerization of fibrous matrices, it enhances formation of capillary-like structures by activated microvascular endothelial cells, whereas LMWH reduces in vitro angiogenesis by rendering matrices less permissive for invasion (6). However, studies of the various effects of both heparins on tumor-associated angiogenesis are limited. A previous study showed that heparins have no effect on growth and angiogenesis of moderately vascularized colon cancer metastases in rat liver (37). In the present study, we compared quantitatively the effects of treatment with UFH, LMWH, or placebo on growth of highly vascularized sarcomas and formation of blood vessels in these tumors in an experimental rat model.

**MATERIALS AND METHODS**

**ANIMALS AND TUMOR MODEL**

A non-immunogenic, rapidly growing osteosarcoma was used that developed spontaneously in rat (2). This well-vascularized tumor is maintained by serial passages in vivo by subcutaneous (s.c.) implantation in Wag-Rij rats. Viable fragments of sarcomas with a diameter of 2-3 mm were implanted s.c. in the right hind limb of 21 male Wag-Rij rats, weighing 130-150 g (Broekman, Someren, The Netherlands). Subsequent growth of the sarcomas was recorded daily by measurement with a calliper, without knowledge of treatment assignment. Tumor volume was calculated as 0.4 x A\(^2\)B, where A is the minimal tumor diameter and B the diameter perpendicular to A. The animal experiments were performed according to guidelines of the Erasmus University, Rotterdam, The Netherlands.

**TREATMENT PROTOCOL**

When the sarcomas had reached a diameter B=5mm, treatment was started. In 2 separate experiments, 3 groups comprising of 7 rats each received either 0.9 % NaCl twice daily intraperitoneally (i.p.), 2.5 mg/kg LMWH (Clivarin, 7000 IU anti-activated X (aXa)/ml; Knoll, Ludwigshafen, Germany) dissolved in 0.9 % NaCl twice daily i.p., or 2.5 mg/kg UFH (5000 IU aXa/ml; Leo, Weesp, The Netherlands) dissolved in 0.9 % NaCl twice daily i.p., respectively, until the animals were sacrificed. These dosages, corresponding to therapeutic plasma concentrations, were based on a previous study, in which aXa plasma levels were determined of UFH-treated and LMWH-treated rats (37). To measure aXa plasma levels, 0.5 ml blood was sampled from the tail vein of all rats on day 4 after treatment was started, at 1, 3 and 6 h after injection, respectively. In the first experiment, rats were sacrificed when tumors had a diameter of B=15mm (SD, 2 mm). Since some tumors reached this diameter already within 7 days, it was decided to continue treatment in the second experiment to 11 days irrespective the size of the tumors, to evaluate effects of prolonged treatment with heparins on tumor growth and angiogenesis.
MEASUREMENT OF AXA PLASMA LEVELS

Blood samples were collected in EDTA, centrifuged (5 min, 10,000 g) and plasma was stored at -80°C until analysis. Plasma levels of the heparins were assayed indirectly by measuring inhibition of factor Xa, using the chromogenic substrate S-2732 (Chromogenix, Molndal, Sweden) as described previously (39). For calibration, UFH and LMWH in normal rat plasma was used.

IN VITRO CYTOTOXICITY ASSAY

Sarcoma cells were plated at concentrations of 1x10^4 cells per well in flat-bottomed 96-well microtiter plates (Costar, Cambridge MA, USA) in a final volume of 0.1 ml of medium per well. Cells were incubated at 37°C in 5% CO2 for 72 h in the presence of various concentrations of UFH or LMWH (test wells) or medium alone (control well) to assess cytotoxicity. Concentrations of UFH and LMWH were between 0 and 50 IU/ml. Growth of sarcoma cells was measured using the Sulphorhodamine-B (SRB; Sigma, St. Louis, MO) assay as described previously by Skehan et al. (33). In short, cells were washed with phosphate buffered saline (PBS), incubated with 10% trichloric acetic acid in distilled water (1h, 4°C) and washed again in distilled water. Cells were then stained with SRB (15-30 min), washed with 1% acetic acid in water and allowed to dry. Protein bound SRB was dissolved in 10 mM Tris buffer, pH 9.4. Absorption was measured at 540 nm. Four replicate experiments were performed. Cancer cell growth was calculated using the formula: cancer cell growth = (test well/control well) x 100 percent.

SAMPLING AND SECTIONING OF THE TUMORS FOR LIGHT MICROSCOPY

Tumors were removed immediately after sacrifice and frozen in liquid nitrogen. The material was kept at -80°C until further use. To evaluate morphology and vessel density of the tumors, 10 sections (8 μm thick) were cut at the site were the tumors had the largest diameter with a motor-driven cryostat with a rotary retracting microtome (Bright, Huntington, UK) at a constant speed and a cabinet temp of -25°C. Sections were mounted on glass slides and stored at -20°C until use.

PREPARATION OF TUMOR SAMPLES FOR ELECTRON MICROSCOPY

Frozen pieces of tumor were fixed in a mixture of 1% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 4 days at 4°C. Then, pieces were rinsed thrice in cacodylate buffer and postfixed in 1% osmium tetroxide (O3O4) in cacodylate buffer, pH 7.4, for 1h at 4°C and dehydrated in a series of alcohol. Sections were embedded in LX-112 epoxy resin. To assess the presence of tissue and the preservation of tissue morphology, 1-2 μm thick sections were studied. Ultrathin sections (40-50 nm) were cut with an LKB Ultratome III and collected on single-hole copper grids carrying a Formvar film. Ultrathin sections were investigated with a transmission Zeiss EM 10c, either unstained or stained with uranyl acetate and lead citrate.
MEASUREMENT OF VESSEL DENSITY IN THE TUMORS

To study morphology of the tumors, sections were stained histochemically using the method of Shoobridge as described previously (20;32). Endothelial cells were detected immunohistochemically with RECA-1, a rat endothelial cell-specific monoclonal antibody (Instruchemie, Hilversum, The Netherlands) (10;37). Sections were air-dried at room temp for 1 h, then fixed for 10 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide and 0.1% sodium azide in PBS for 15 min. Sections were incubated for 60 min with RECA-1 diluted 1:10 in PBS containing 0.2% (w/v) bovine serum albumin, then for 60 min with rabbit anti-mouse IgG peroxidase (Dako, Glostrup, Denmark) diluted 1:200 in PBS in the presence of 0.2% bovine serum albumin and 5% normal rat serum. Sections were stained for peroxidase activity using 3,3-diaminobenzidinetetracloride and hydrogen peroxide (34). Analysis of vessel density was performed with a Vanox-T-photomicroscope (Olympus, Tokyo, Japan) x5 objective (N.A. 0.08). Images of sections were captured using a CCD camera (4913 Cohu, San Diego, CA) that was attached to a frame grabber (Scion Image 1.59 for Mac; Scion, Frederick, MD) and a computer (8100 Apple Macintosh, Cupertino, CA). Vessel density was determined as the ratio of the area of RECA-1-positive endothelial cells and the total area of the section (x 100) ± standard error of mean (SEM). Vessel density was measured in 2 sections of each tumor and values were averaged to determine vessel density per tumor. Analysis of vessel density was performed without knowledge of the treatments given.

STATISTICAL ANALYSIS

The slopes of the best fitting curves of tumor volumes versus time after logarithmic transformation was taken as a measure for the growth rate. ANOVA was used to analyse differences between groups of rats with respect to growth rates of the tumors and tumor vessel density. Values of p < 0.05 were considered to indicate significant differences.

RESULTS

aXa PLASMA LEVELS

aXa plasma levels of placebo-treated, UFH-treated and LMWH-treated rats in the first experiment are presented in Fig. 1. High plasma levels (>1.0 aXa IU/ml) were found for at least 3 h after administration of either UFH or LMWH, whereas therapeutic plasma levels (>0.3 aXa IU/ml) were present for almost 6 h after administration. No bleeding complications were observed throughout the experiments.
Growth rate of tumors

Results of experiments 1 and 2 are presented in Fig. 2. In the first experiment, a high variation in initial volume of the tumors was observed. Correlations were not observed between initial volumes of the sarcomas and their growth rate as can be deduced from the slopes of the curves shown in Fig. 2. In experiment 2, the initial volumes of the sarcomas had been controlled more rigidly. In this experiment, 1 rat in the LMWH-treated group died during blood sampling. Treatment of the rats with either UFH or LMWH did not affect growth of the implanted tumors in both experiments (p = 0.4). Metastases were not found in any of the rats irrespective of treatment.

Figure 1. αXa plasma levels (± SEM) of placebo-treated (●—●) (n=7), UFH-treated (□—□) (n=7), and LMWH-treated (X—X) rats (n=7) after 4 days of treatment.

Figure 2. Volumes of tumors in time, grown in placebo-treated (●—●), UFH-treated (□—□), and LMWH-treated (X—X) rats in experiment 1 and 2. The slopes of the best fitting curves of tumor volume versus time after logarithmic transformation of the data was taken as a measure for the growth rate. Statistically significant differences between the groups of rats were not found with respect to growth rates of the tumors (p = 0.4).
MORPHOLOGY AND VESSEL DENSITY OF SARCOMAS

Sarcomas mainly consisted of cancer cells. Stroma or extracellular matrix was scarcely present in the tumors (Fig. 3A, B). Sarcomas in all treatment groups were well-vascularized (Fig. 3B, C). Mean vessel density of tumors in placebo-treated rats was 11.2%. In UFH-treated and LMWH-treated rats the mean vessel density was 11.2% and 10.8%, respectively. Again, significant differences were not found between the rats in the 3 treatment groups (p=0.7; Table 1).

Figure 3. Photomicrographs of cryostat sections (A, C) and ultrathin sections (B) of rat sarcomas. (A) Sections were stained with the Shoobridge method (20:32) or (B) visualized with electron microscopy to demonstrate cancer cells and stroma. Sarcomas mainly consisted of cancer cells whereas stroma or extracellular matrix was scarcely present. Endothelial cells (e) of vessels were present in between sarcoma cells (c). m, mast cell. (C) RECA-1 was used to detect endothelial cells immunohistochemically (10:37). Endothelial cells are darkly stained. Sarcomas in all treatment groups were highly vascularized. Bar = 50 μm (A), 5 μm (B) and 100 μm (C).
In vitro cytotoxicity assay

The in vitro dose/responses of sarcoma cells to either UFH-treatment or LMWH-treatment are shown in Fig. 4. The heparins were not cytotoxic to the sarcomas cells up to concentrations of 50 IU/ml.

**Figure 4.** In vitro cytotoxicity of UFH (A) or LMWH (B) on rat-osteosarcoma cells after 72 h of incubation in the presence of various concentrations of the heparins. Cytotoxicity is expressed as percentage of the number of cells present after treatment with heparins and the number of cells present after treatment with medium alone. Mean values of 4 bioassays in duplicate are shown ± SEM.

Discussion

The observations in recent clinical studies that UFH and LMWH have different effects on survival of patients with cancer have initiated a debate whether heparins interfere with cancer progression (17). A number of experimental studies have shown that heparins can alter metastasis or related processes such as angiogenesis (for reviews, see (11;36)). UFH can stimulate blood vessel formation, and both in vitro and in vivo studies have shown that LMWH can inhibit angiogenesis (6;19;24;28;38). However, in the present quantitative study we did not observe any effect of either UFH or LMWH on growth of sarcomas or blood vessel formation within the tumors. These findings are in agreement with our previous study, in which we did not find an effect of both heparins on CC531s rat colon carcinoma metastasis and vessel density of the tumors in livers of rats (37). The lack of effects of UFH and LMWH cannot be explained by too low plasma concentrations of the heparins. Therapeutic levels of both UFH and LMWH were present in plasma of the rats for almost 6 h after administration, with even supra-therapeutic levels in the first hours. In the other studies in which effects of heparins were observed, equivalent dosages of heparins significantly affected either experimentally induced metastasis or angiogenesis in rats (7;14;22;29;43). In some of these experiments only a single injection of heparin was administered before inoculation of cancer cells (7;22;43), whereas we continued treatment until sacrifice. In contrast to the CC531 colon cancer metastases, the osteosarcomas used in the present study were highly vascularized with approx. 11% of the tumor volume consisting of vessels (Table 1). Although it is possible that the
sarcomas grew too fast and were too highly vascularized to reveal subtle effects of treatment, previous studies using similar models have shown that growth of rat sarcomas, as well as neovascularization of the respective sarcomas, can be significantly reduced by angiogenesis-inhibitors (8;13;21).

Hence, on the basis of the present results, we like to conclude that the effects of heparins on tumor-associated angiogenesis \textit{in vivo} are limited. Other \textit{in vivo} studies evaluating effects of UFH on growth of s.c. implanted tumors of various origins support this conclusion (9;15;16;22;23;26;30;44). In these studies, UFH did not affect primary tumor growth either, but rather spontaneous metastasis of the implanted tumors. However, these effects were not equivocal because UFH enhanced metastasis in some of the studies (15;16;30), whereas in others metastasis was inhibited by UFH treatment (22;23;26;44).

The potential effects of UFH and probably also of LMWH on cancer progression are numerous. Besides the anticoagulant function of heparins and the previously mentioned effects on angiogenesis, UFH can alter proliferation (3), adhesion (27) and migration (1;25) of cells, and is able to modulate activity of proteolytic enzymes which play major roles during invasion and metastasis (5;18). Since processes such as proliferation, migration, invasion and angiogenesis are important for both tumor growth and metastasis, it is difficult to understand why UFH affects metastasis but not growth of primary tumors \textit{in vivo}. Moreover, the numerous biological activities of UFH and the conflicting findings in previous animal studies do not support the hypothesis that UFH affects cancer progression in one direction or by one specific mechanism. Therefore, data from \textit{in vitro} experiments or animal experiments on effects of UFH on cancer progression or related processes should be interpreted with caution. Effects may well be specific for the tumor type or model, or may be a consequence of one particular activity of UFH, rather than a reflection of the overall effect of UFH on cancer progression. A recent systematic clinical review of the effects of UFH versus placebo or no treatment on survival of patients with malignancy supports the assumption that the ultimate effect of UFH on cancer progression is probably minimal (35).

The effects of LMWH on cancer are less well studied than UFH. At present, clinical trials are being performed to study effects of LMWH on survival of cancer patients, both in patients with and without concurrent venous thromboembolism.

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