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Selective antimetastatic effect of heparins in preclinical human melanoma models is based on inhibition of migration and microvascular arrest

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Abstract

Use of heparin derivatives in several cancer types revealed that anticoagulant therapies have a beneficiary side effect: delay of tumor progression. Since there are no data on human melanoma, we have analyzed the effect of heparins in preclinical models. Neither unfractionated heparin (UFH), nor its low molecular weight derivative (LMWH) influenced *in vitro* or *in vivo* growth of HT168-M1 human melanoma cells. However, heparins significantly inhibited lung colony formation and liver metastasis development in the concentration range of 20–200 IU/kg, whereas recombinant hirudin was ineffective. The antimetastatic effect was due to an early (5–60 min) inhibition of tumor cell arrest in the lung microvasculature. Analysis of the molecular mechanism of the antimetastatic effect of heparins indicated a specific inhibition of tumor cell migration and matrix invasion. The presented experimental data suggest that heparins have specific antimetastatic effect in the case of human melanoma, which is independent from the coagulation cascade.

Introduction

Malignant melanoma is one of the most metastatic human cancers, where even a few millimeter thick tumor may have a high risk for developing distant metastasis within 5 years. Unfortunately, this tumor type is highly resistant to chemotherapy; therefore, pharmacological interventions are urgently needed to improve patient survival.

Most tumor types, especially in advanced stage of the disease, are characterized by coagulation disorders, due to a prothrombotic state [1]. Tumor cells produce various procoagulants, which are responsible for hypercoagulation via activating the extrinsic coagulation pathway and factor X, ultimately leading to the production of thrombin (factor IIa) [2]. Accordingly, anticoagulant therapy of cancer patients with thromboembolic complications became a standard procedure using mostly coumarin derivatives and/or heparins. Analysis of clinical data revealed that heparins are superior over other anticoagulants in managing cancer-induced thromboembolism where low molecular weight heparins (LMWH) outperformed the classical form [3, 4]. Moreover, clinical trials proved that the use of warfarin [5–7] but more frequently heparin-derivative treatments of cancer patients delayed tumor progression and prolonged survival [8–11].

In the past 40 years, a large amount of experimental data provided evidences for the antimetastatic effect of heparins in various rodent metastasis models: the most conclusive ones came from studies on melanoma and mammary carcinoma [12]. Recently, the molecular mechanisms of the selective antimetastatic effect of heparin were revealed when a series of studies provided evidences for the involvement of platelet and endothelial P- and leukocyte L-selectins in the effect of heparins [13–15].

Since heparins were shown to be antimetastatic in rodent melanoma, but there are no data on human melanoma, the aim of this preclinical study was to test the antimetastatic effect of heparins in a human melanoma model.

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Materials and methods

In vitro *culture*

HT168-M1 human melanoma cell line was maintained *in vitro* as monolayer culture in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5% fetal calf serum (FCS, Sigma) and 1% penicillin–streptomycin solution (5000 U/ml penicillin and 5 mg/ml streptomycin, Sigma) at 37 °C in a 5% CO_2 atmosphere.

Anticoagulants

Two types of heparins were used: unfractionated heparin-natricum (UFH) (M_w: 12–15.000 Da, Richter Co., Budapest, Hungary) and low molecular weight

dalteparin-natricum (LMWH, M_w: 5000 Da, Fragmin, Pharmacia/Pfizer, Stockholm, Sweden). As control anticoagulant, recombinant hirudin (Mw: 6979.5 Da, Refludan, Hoechst Marion Roussel GmbH, Frankfurt am Main, Germany) was used in 0.9% NaCl solution.

Cell proliferation assay

Two thousand tumor cells per well were plated in flat-bottomed, 96-well tissue culture plates in RPMI-1640 medium containing 5% FCS, and after 24 h cells were treated with 0.01–1 IU/ml UFH or LMWH for 72 h at 37 °C. At the end of incubation a colorimetric assay (MTT, Sigma) was performed [16]. The absorbance at 570 nm was measured with microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) in 6 parallel samples.

Cell adhesion to extracellular matrix (ECM) proteins in vitro

The wells of 96-well plates were coated with 5 μg/well of ECM proteins overnight at 4 °C: fibronectin, laminin (Sigma), Matrigel (Collaborative Research, Bedford, MA), collagen I–III (from rat tail), fibrinogen (Fraction I from human plasma, Sigma), all diluted in 50 μl PBS. Tumor cells were pretreated with 0.1–1 IU/ml of UFH or LMWH for 1 h at 37 °C. After treatment, 2×10^4 tumor cells/well were allowed to adhere to the matrix in 96-well plates for 35–60 min in serum-free medium (SFM). Non-adherent cells were then discarded and the ratio of the attached cells – compared to control wells containing all plated cells – were determined by staining with Sulforhodamine B [16].

Melanoma cell adhesion to endothelial cells in vitro

Twelve-well slides (ICN Biomedicals Inc., Aurora, OH, USA) were coated with fibronectin (50 μg/ml, Sigma) diluted in PBS for 1 h at 37 °C. Human endothelial-like cells (KS-Imm) [17] (10^5/well) in RPMI medium containing 5% FCS were allowed to adhere to FN for 2 h at 37 °C. Confluent KS-Imm cells were covered with heparin-pretreated (1 IU/ml UFH or LMWH) HT168-M1 human melanoma cells (10^4/well) diluted in SFM for 60 min at 37 °C. Cells were fixed with 4% paraformaldehyde (PFA), and immunohistochemistry was performed after permeabilization with 0.2% saponin. A 1:1 mixture of monoclonal anti-gp100 (HMB45, DAKO, Glostrup, Denmark; diluted 1:100 in PBS) and anti-MART-1 (M2-7 C10 hybridoma supernatant; diluted 1:10 in PBS) antibodies was used for the detection of melanoma cells, and PBS for negative control. Biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA, USA) was used as secondary reagent, followed by streptavidin–peroxidase treatment (DAKO). Antibody binding was visualized with 3-amino-9-ethylcarbazole (AEC, Shandon, Nottingham, UK), then the slides were counterstained with hematoxylin. Adherent melanoma cells were counted under light microscope (200× magnification), and data have been statistically analyzed in 4 parallel samples.

Cell migration and invasion through Matrigel in vitro

The migration and invasion assays were performed in a 96-well CXF8 plate (polycarbonate filter with 8 μm pore size, Neuroprobe Inc., Cabin John, MD, USA). The filters were coated with PBS (migration assay) or with Matrigel (50 μg/ml, invasion assay) overnight at 4 °C. The lower chamber of the CFX8 plate was filled with 30 μl of 10% FCS (as attractant) diluted in RPMI. Tumor cells were pretreated for 30 min with 1 IU/ml UFH or LMWH. Cells were then suspended in RPMI, and 20 μl of cell suspension was added to the top of the filter (2×10^4/well). Migration was allowed to proceed for 20 h at 37 °C. After incubation, cells in the upper chamber were wiped off, the migrating cells attached to the filter were fixed in methanol for 5 min and stained with 1% toluidine-blue for 5 min. The number of migrated tumor cells (in 6 parallel samples) was counted using light microscope in three separate fields for each well with 400× magnification and analyzed statistically.

Animal experiments

All experimental protocols were carried out in accordance with the Guidelines for Animal Experiments [18], and were approved by the Ethics Committee of the Institute.

Lung colonization assay

Female BALB/c SCID mice at the age of 2–3 months were used. Single-cell suspension was prepared from 0.02% EDTA-treated monolayer of HT168-M1 cultures, washed and diluted in SFM. Tumor cells (5×10^5 or 10^6 per animal) in 200 μl SFM were injected intravenously into the tail vein of anaesthetized mice. UFH, LMWH (20–200 IU/kg diluted in 100 μl of 0.9% NaCl per each animal) and hirudin (3 mg/kg) were administered intraperitoneally daily from the day before tumor inoculation till the 3rd day after tumor cell inoculation. Controls received 100 μl of saline. Animals were followed for 7 weeks and sacrificed by Nembutal (Serva, Heidelberg, Germany) overdose on day 50, when either the weight loss reached a maximum of 20%, or the animals in control groups showed hyperventilation. Lungs were removed, fixed in 4% PFA, and surface tumor colonies were counted under stereo light microscope.

Dynamics of tumor cell seeding in the lung

Female SCID mice were treated with heparins (200 IU/kg) or saline as control, i.p. 1 day before tumor cell inoculation. One million tumor cells were injected i.v. and animals were terminated with Nembutal (Serva) overdose after 5 min, 1 or 4 h. The lungs were removed,

fixed in PFA, embedded in paraffin, and 3 μm sections were used in the studies. Immunohistochemistry was performed after microwave antigen retrieval. Endogenous peroxidase activity was blocked with incubation of the slides in 3% H_2O_2 in methanol, and non-specific binding sites were blocked with 20% goat serum. Monoclonal HMB45 and anti-MART-1 antibodies were used as above. Human melanoma cells were counted under light microscope (400× magnification) and data have been statistically analyzed in 3 parallel samples.

Liver metastasis assay

Tumor cells (10^5) were injected into the spleen in a volume of 20 μl into female SCID mice under Nembutal anaesthesia. Daily treatment with UFH and LMWH (20–200 IU/kg), hirudin (3 mg/kg) or saline as control, was started on the 8th postinjection day and continued for 2 weeks. Animals were sacrificed by Nembutal overdose on day 31, when in control groups the weight loss reached a maximum of 20%. The spleens and livers were removed, measured and fixed in 4% PFA. Surface liver metastases from 10 animals/group were counted under stereo light microscope.

Measurement of coagulation parameters

For measurement of the anticoagulant effect of heparins, the most widely used laboratory parameter is activated partial thromboplastin time (aPTT). Normal range referred by our laboratory is between 22 and 38 s. Beside aPTT we measured thrombin time (TT), the level of prothrombin, International Normalized Ratio (INR) and level of fibrinogen (normal ranges: 12–19 s, 80–120%, 0.9–1.2, and 200–400 mg/dl, respectively) in the blood of treated SCID mice. One day or one hour before blood taking in each experimental group, 3-3 female SCID mice were treated i.p. with UFH or LMWH (20–200 IU/kg), hirudin (3 mg/kg) or NaCl solution. Mice were anaesthetized by i.p. administration of Nembutal and blood was taken from the heart. Fresh blood was kept in glass tubes in 3.8% sodium citrate solution (citrate:blood ratio = 1:10 v/v). The samples were centrifuged for 15 min at 4000 rpm and the coagulation parameters were determined in SYSMEX CA 1500 coagulometer.

Detection of circulating melanoma cells

One milliliter cardiac blood was drawn from Nembutal-anasthetized animals 5–60 min following tumor cell injection i.v., the sample was treated with EDTA and nucleated cells were isolated with Ficoll–Hypaque (Sigma). Total cellular RNA was extracted from nucleated cells using Trizol (Sigma) reagent according to the instructions of the manufacturer. One μg of total RNA was reverse transcribed using oligo(dT) primer (Invitrogen) and M-MLV reverse transcriptase (Sigma). The reaction mixture was incubated at 37 °C for 50 min, heated at 85 °C for 20 min, and then stored until use at –20 °C. The quantitative PCR analysis for mRNA expression of human WT1 was standardized by co-amplifying this gene with the housekeeping human gene, β-actin. The real-time PCR reaction was run on the iCycler iQ™ (Bio-Rad) using standard conditions, namely optimized concentration of primers (final concentration 200 nM), IQ™SYBR Green Supermix (containing 100 mM KCl, 40 mM Tris–HCl, pH 8.4, 0.4 nM of each dNTP, 6 mM $MgCl_2$, 50 U/ml iTaq DNA polimerase, SYBR Green I, 20 nM fluorescein) and 2 μl cDNA. A no template control (containing water) was used as negative control for every different primer pair. The starting quantity of gene expression in the sample was determined by comparison of unknown to a standard curve generated from a dilution series of template DNA of known concentration.

Statistical analysis

Data from *in vitro* experiments and from morphometric analyses were compared using Student's *t*-test; *P*-values < 0.05 were considered significant. The *in vivo* data were compared using Mann–Whitney U test. All statistical analyses were performed using CSTAT statistical software program.

Results

Effects of anticoagulants on lung and liver metastatization of human melanoma cells

HT168-M1 melanoma cells were injected i.v. into anticoagulant-pretreated SCID mice which were further treated for 3 days. At termination (on day 50), results showed that heparins at low (20 IU/kg) or human equivalent dose (200 IU/kg) inhibited lung metastasis formation (Figure 1a). This effect was specific for heparins, since recombinant hirudin did not affect lung colonization at human equivalent dose (3 mg/kg, Figure 1b). Moreover, LMWH treatment not only reduced the number of lung colonies, but also reduced the incidence of metastases unlike UFH or hirudin (Figure 1b).

The anticoagulants were tested in a liver metastasis model as well. Animals were treated from the 8th postinjection day for another 2 weeks i.p. Heparins at low and at human equivalent dose effectively and significantly inhibited liver metastasis formation (Figure 1c). Similarly to lung colonization, hirudin treatment did not influence liver metastasis formation (Figure 1c).

Effect of anticoagulants on the hemostasis of SCID mice

One day and 1 hour before blood taking, SCID mice were treated i.p. with UFH, LMWH or hirudin. We have measured the most important blood coagulation

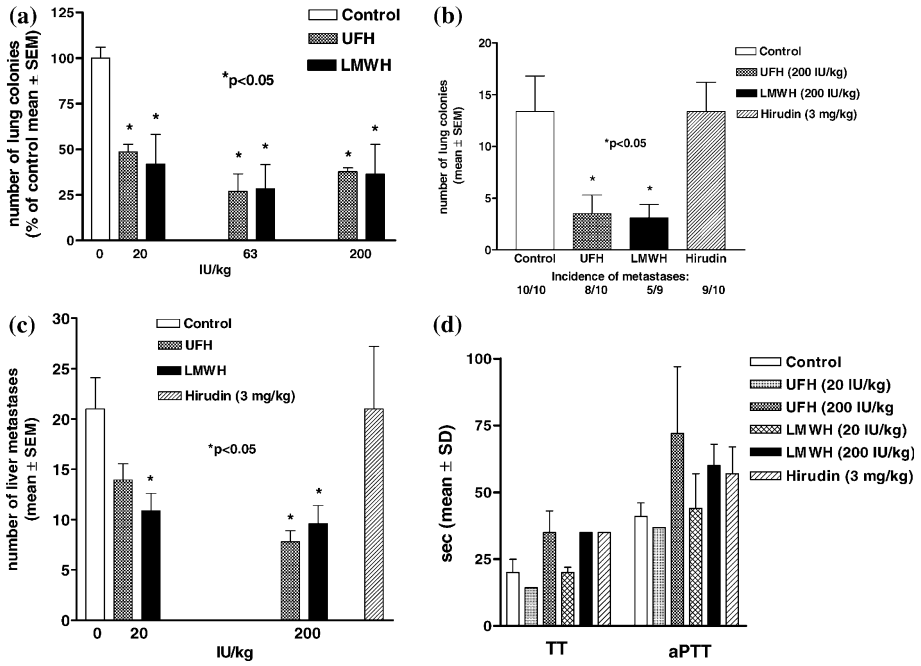

Figure 1. Effect of heparins on metastatization of tumor cells in SCID mice, and on hemostasis. (a, b) Lung colony assay (mean ± SEM of 3 separate experiments, $n = 10$), (c) Liver metastasis assay ($n = 10$), (d) Hemostasis parameters ($n = 3$), TT: thrombin time, aPTT: activated partial thromboplastin time.

parameters: aPTT, TT, as well as prothrombin and fibrinogen levels were measured. Following heparin and hirudin therapies the aPTT and TT levels increased at the human equivalent dose (200 IU/kg and 3 mg/kg, respectively), and there were no significant differences between the anticoagulants in this respect (Figure 1d). Prothrombin and fibrinogen levels of SCID mice did not change following pretreatment (data not shown).

Tumor cell proliferation in vitro *and* in vivo

None of the heparins affected melanoma cell growth *in vitro* (Figure 2a), or intrasplenic tumor growth *in vivo* (Figure 2b), and hirudin was also ineffective in this respect (data not shown).

Interaction of tumor cells with ECM components and endothelial cells in vitro

For determining the effect of heparins on tumor cell adhesion to ECM proteins, melanoma cells were incubated on plates coated with ECM proteins: Matrigel, fibrinogen, fibronectin, laminin and collagen I–III for 35–60 min, and the percentage of adherent cells was determined. Heparins (0.1–1 IU/ml) did not affect the adhesion potential of human melanoma cells to the matrix proteins used (Figure 3a, see data for FBG and Matrigel exclusively).

We have also studied the adhesion of melanoma cells to endothelial cell monolayer *in vitro*. Melanoma cells were allowed to adhere to endothelial cells for 60 min, and adhered cells were identified using HMB45/anti-MART-1 antibodies. We found that heparins (1 IU/ml) significantly

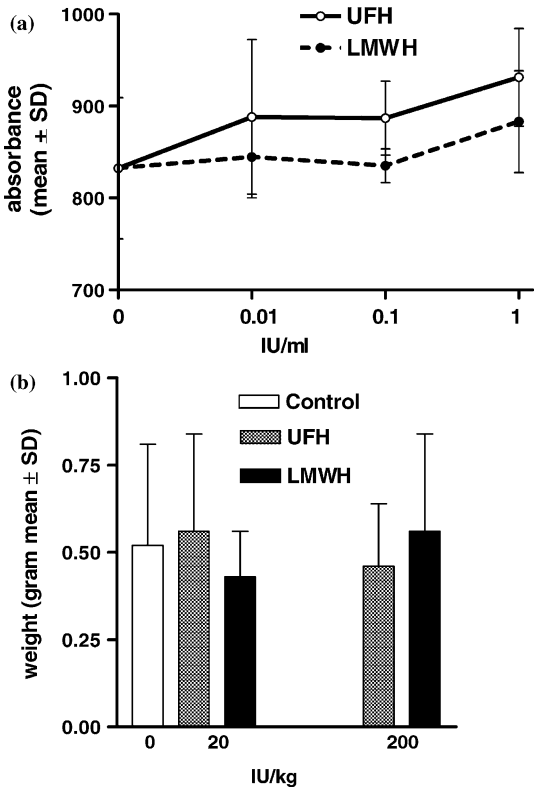

Figure 2. Effect of heparins on *in vitro* and *in vivo* growth of human melanoma cells. (a) MTT assay ($n = 6$), (b) Primary tumor weight (spleen, $n = 10$). (a, b) 0 represents solvent control.

inhibited melanoma cell adhesion to endothelial cells (Figure 3b), confirming previous observations [15].

Migration of melanoma cells was also tested after treatment with heparins (1 IU/ml). We found that UFH

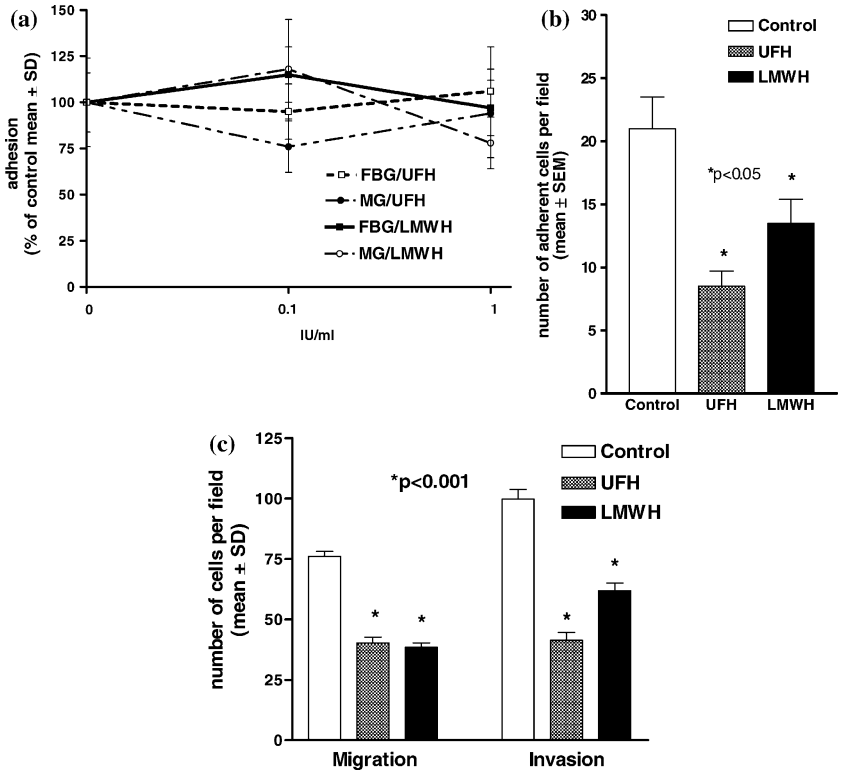

Figure 3. Effect of heparins on the matrix and endothelial cell adhesion as well as migration and Matrigel invasion of human melanoma cells *in vitro*. (a) Matrix adhesion for 60 min ($n = 6$), FBG: fibrinogen, MG: Matrigel, (b) Endothelial cell adhesion for 60 min ($n = 4$), UFH and LMWH:1 IU/ml, (c) melanoma cell migration for 20 h at 37 °C ($n = 6$), UFH and LMWH:1 IU/ml, attractant:10% FCS.

and LMWH pretreatments significantly abrogated melanoma cell migration (Figure 3c). Next we studied Matrigel invasion of melanoma cells, and found that heparins significantly inhibited matrix invasion of human melanoma cells as well (Figure 3c).

Analysis of the antimetastatic effect in vivo

Lung colonization assay recapitulates the second phase of hematogenous metastasis formation, from their circulating state to extravasation and colonization. We have questioned which phase of this complex process is affected by the treatment with heparins. For this purpose, we studied the dynamics of melanoma cell seeding in the lung of SCID mice directly after intravenous injection. Mice were pretreated with the human equivalent dose of UFH and LMWH (200 IU/kg) one day or 1 h before tumor cell inoculation, and were terminated at 5 min, 1 h or 4 h following tumor cell injection. Tumor cells were identified in the lung tissue by the expression of melanoma markers and counted under microscope. Even at the earliest time point (5 min) the density of tumor cells in the lung capillaries was significantly reduced in heparin-pretreated animals to ~40% of the control, which effect was maintained for 1 h (Figures 4a–c).

The effect of the heparin pretreatment on the amount of circulating viable tumor cells was measured by a sensitive quantitative PCR technique determining the mRNA expression of the human WT1 gene [17]. Cardiac blood was drawn from pretreated animals 5 and 60 min following i.v. injection of tumor cells. Data indicated that in the animals pretreated with heparins (200 IU/kg) the number of circulating tumor cells was much higher at the earliest point studied (5 min), while this difference disappeared at 60 min (Figure 4d), when a significantly reduced number of circulating viable human tumor cells could be found in the circulation.

Discussion

In this work we have shown for the first time that heparins (both UFH and LMWH) have antimetastatic effect in human melanoma metastasis models. Previous studies provided evidence for antimetastatic potential of heparins in murine melanoma models [12], which was confirmed in this study. Looking for possible molecular mechanisms of this effect, recent experimental data pointed to the involvement of P- and L-selectins, tissue factor pathway inhibitor (TFPI) released from endothelial cell, and the tumor cell–host interactions [13, 19]. Experimental studies suggested that the interactions of tumor cells (including melanoma) with platelet- and endothelial P-selectin molecules were responsible for the antimetastatic effects of heparin, since in P- or L-selectin deficient animals the activity disappeared [13–15].

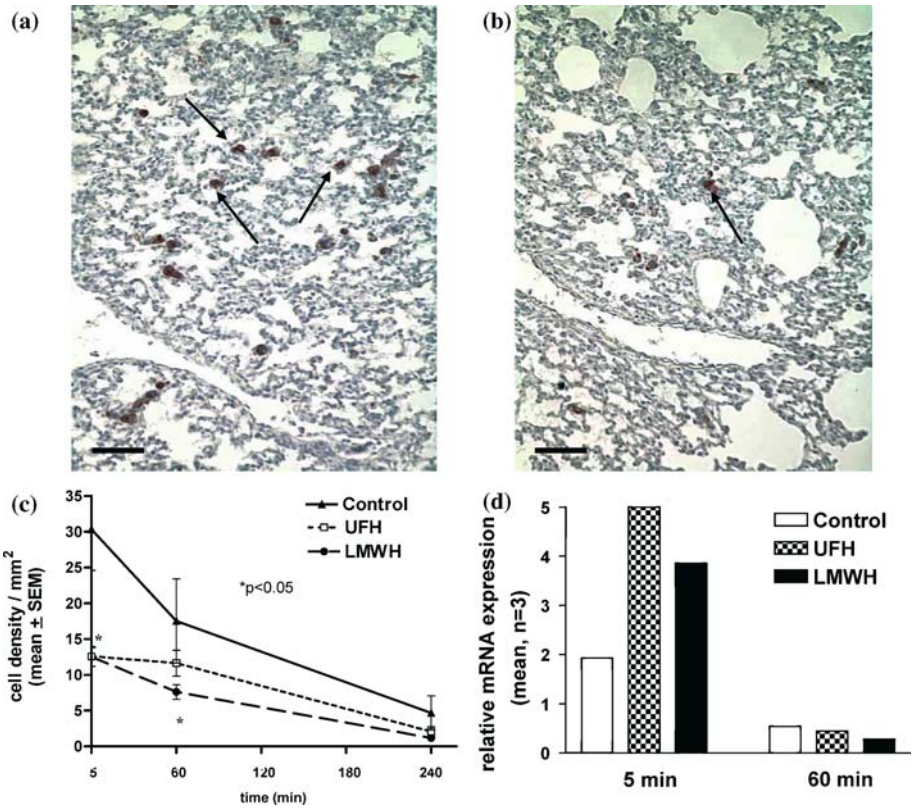

Figure 4. Effect of heparins on the arrest of melanoma cells in microvessels of the lung (5 min–4 h). (a, b) Immunohistochemistry (HMB45/anti-MART-1) of human melanoma cells in the lung of SCID mice 5 min following i.v. injection, light microscopy. Bar: 100 μm, arrows: melanoma cells. (a) Control, (b) 200 IU/kg LMWH. (c) Dynamics of melanoma cell arrest in the lung (n = 3), morphometry. UFH and LMWH: 1 IU/ml. (d) Detection of human melanoma cells in the circulation of SCID mice by WT1 qPCR analysis (n = 3).

It has been considered that the progression-inhibitory effect of heparins is due to tumor cell interactions with platelets and the coagulation system. In our metastatic human melanoma model neither of these seem to be involved in the antimetastatic effects of heparins. First, the antimetastatic effect is detectable at doses lower than the human-equivalent therapeutic dose, where the coagulation-modulatory effects are not yet detectable. Second, the human melanoma cell line used in this study (HT168-M1) does not aggregate platelets *in vitro* (data not shown), similarly to a murine model where heparins were active [19]. Moreover, in contrast to previous data, recombinant hirudin had no antimetastatic effect in our experimental model, suggesting the lack of involvement of thrombin. The metastasis promoting potential of thrombin was demonstrated in the lung colonization of experimental melanoma model [20, 21]. Thrombin effect is due to the ectopic and functional expression of thrombin receptor (PAR) shown to be present in various tumor cell types, including melanoma, found to be involved in invasion and metastasis [22–24]. However, there is no data on functionally active PAR receptor expression in HT168-M1 human melanoma cell line, which may result in the lack of the effect of hirudin (and tumor cell-induced platelet aggregation potential). Furthermore, out of four tumor types, only in one (lung cancer) was the disease progression found to be dependent on thrombin, and, consequently, sensitive for antithrombin therapy [25].

Another possible mechanism of the antimetastatic effect of heparins in human melanoma xenografts could be the L-selectin-mediated effects inhibiting interactions with leukocytes [26, 27]. Morphological analysis of the early phases of lung colonization did not reveal such cells in the close vicinity of human melanoma cells in the alveolar capillaries, excluding this option. Analysis of the antimetastatic effect of heparins revealed that injected human melanoma cells are retained in the circulation, exposing tumor cells to mechanical stress for a longer period. On the other hand, heparin pretreatment inhibited arrest of human melanoma cells in the lung capillaries, contributing to the observed antimetastatic effect. This effect was corroborated by our data that human melanoma cell adhesion to endothelial cells was inhibited by heparins *in vitro*, similarly to previous reports [15].

It was shown in various experimental models that heparins have an antiangiogenic effect as well [28]. In our study, however, heparin treatment of SCID mice had an early inhibitory effect on the entrapment of tumor cells in the lung capillaries (within 4 h), making improbable that the inhibition of angiogenesis could play a significant role in the antimetastatic effect of heparins in human melanoma xenografts. Furthermore,

lung is among the best vascularized organs, where cancer cells generally do not need to induce new vessels at the early phase of metastatization.

Our *in vitro* studies on a direct antimelanoma effect of heparins revealed an interesting new potential of these carbohydrates, a specific antimigratory effect, without interfering with any other *in vitro* activities of tumor cells studied: proliferation, survival or matrix adhesion. Data in the literature indicate that the P-selectin-mediated antimetastatic effect of heparin is due to the glycosaminoglycan chains of the tumor cell surface heparan sulfate proteoglycans (HSPGs) [29]. In human melanoma CD44v3 and syndecan-4 HSPGs have been shown to be expressed and involved in tumor progression [30, 31]. Experimental studies demonstrated that both the inhibition of heparan sulfate biosynthesis or interference with its functions using basic peptides have significant antimetastatic effect in human melanoma models [32, 33]. Heparins may compete with the binding of these surface sugar moieties to either ECM or cytokines. Extravasation of tumor cells is a critical event during organ colonization, where migratory activities of tumor cells play a critical role. It is of note from this respect that heparins specifically inhibit melanoma cell migration and invasion in artificial basement membrane. Potential molecular mechanism of this inhibition could be the interference with the integrin-supportive functions of heparan sulfate proteoglycans [27], or even with the integrins themselves. Data indicate that heparin can directly bind αIIbβ3, the predominant integrin of platelets [34]. We have shown before that several tumor cell types, including human melanoma, express ectopic αIIbβ3 integrin [35], and the interference with its functions by antibodies specifically abrogates melanoma cell migration *in vitro* and attenuates metastatic potential *in vivo* [36].

Recently two forms of individual cancer cell migration were identified, the mesenchymal type, dependent on integrins, and the amoeboid type, independent of proteolysis and integrins [37]. The latter form was suggested to be mediated by matrix glycosaminoglycans [38]. Our data on the predominant heparin-sensitive migration of human melanoma cells fits well into the glycosaminoglycan-dependent amoeboid migration model.

The observed *in vivo* effect of heparins could be exploited clinically in the management of advanced stage melanoma. The observation that low-dose heparins have similar antimetastatic effect on human melanoma than the human-equivalent dose is promising, since it allows the administration of these drugs for an extended period of time (for months) after the detection of early stage of progression (either lymphatic or skin recurrence). A few clinical studies already provided data on the direct antimetastatic effect of LMWH in the case of breast and pelvic cancers [9, 39], but not in melanoma. Considering melanoma as a relatively chemoresistant tumor, LMWH therapy of the progressing disease may have clinical significance.

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References

1. Lip GYH, Chin BSP, Blann AD. Cancer and the prothrombotic state. Lancet Oncol 2002; 3: 27–34.
2. Letai A, Kuter DJ. Cancer. Coagulation and anticoagulation. Oncologist 1999; 4: 443–9.
3. Green D, Hull RD, Brant R et al. Lower mortality in cancer patients treated with low-molecular-weight *versus* standard heparin. Lancet 1992; 339(8807): 1476.
4. Lee AYY, Levine MN, Baker RI et al. Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. N Engl J Med 2003; 349: 146–53.
5. Zacharski LR, Henderson WG, Rickles FR et al. Effect of warfarin on survival in small cell carcinoma of the lung. JAMA 1981; 145: 831–5.
6. Zacharski LP, Henderson WG, Rickles FR et al. Effect of warfarin anticoagulation on survival in carcinoma of the lung, colon, head and neck, and prostate. Cancer 1984; 53: 2046–52.
7. Chahinian AP, Propert KJ, Ware JH et al. A randomized trial of anticoagulation with warfarin and of alternating chemotherapy in extensive small-cell lung cancer by the cancer and leukemia group B. J Clin Oncol 1989; 7: 993–1002.
8. Lebeau B, Chastang C, Brechot J-M et al. Subcutanous heparin treatment increases survival in small cell lung cancer. Cancer 1994; 74: 38–45.
9. von Tempelhoff GF, Harenberg J, Niemann F et al. Effect of low molecular weight heparin (Certoparin) versus unfractionated heparin on cancer survival following breast and pelvic cancer surgery: A prospective randomized double-blind trial. Int J Oncol 2000; 16: 815–24.
10. Constantini S, Kanner A, Friedmann A et al. Safety of perioperative minidose heparin in patients undergoing brain tumor surgery: a prospective, randomized double-blind study. J Neurosurg 2001; 94: 918–21.
11. Robert F, Busby E, Marques MB et al. Phase II study of docetaxel plus enoxaparin in chemotherapy naïve patients with metastatic non-small cell lung cancer: Preliminary results. Lung Cancer 2003; 42: 237–45.
12. Smorenburg SS, van Noorden CJF. The complex effects of heparins on cancer progression and metastasis in experimental studies. Pharmacol Rev 2001; 59: 93–105.
13. Borsig L, Wong R, Feramisco J et al. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA 2001; 98: 3352–7.
14. Borsig L, Wong R, Hynes RO et al. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci USA 2002; 99: 2193–8.
15. Ludwing RJ, Bochme B, Podda M et al. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res 2004; 64: 2743–50.

16. Martin M, Clynes M. Comparison of 5 microplate colorimetric assays for in vitrocytotoxicity testing and cell proliferation assays. Cytotechnology 1993; 11: 49–58.
17. Rásó E, Mészáros L, Albini A et al. A WT1 expressing metastatic human Kaposi sarcoma xenograft model. Pathol Oncol Res 2004; 10: 22–5.
18. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR). Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). Br J Cancer 1998; 77: 1–10.
19. Amirkoshravi A, Mousa SA, Amaya M et al. Antimetastatic effect of tinzaparin, a low-molecular-weight heparin. J Thromb Haemost 2003; 1: 1972–6.
20. Nierodzik ML, Plotkin A, Kajumo F, Karpatkin S. Thrombin stimulates tumor-platelet adhesion *in vitro* and metastasis *in vivo*. J Clin Invest 1991; 87: 229–36.
21. Esumi N, Fan D, Fidler IJ. Inhibition of murine melanoma experimental metastasis by recombinant desulfatohirudin, a highly specific thrombin inhibitor. Cancer Res 1991; 51: 4549–56.
22. Booden MA, Eckert LB, Der CJ, Trejo J. Persistent signaling by dysregulated thrombin receptor trafficking promotes breast carcinoma cell invasion. Mol Cell Biol 2004; 24: 1990–9.
23. Darmoul D, Gratiuo V, Devaud H et al. Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. Am J Pathol 2003; 162: 1503–13.
24. Tellez C, McCarty M, Ruiz M, Bar-Eli H. Loss activator protein-2alpha results in overexpression of protease-activated receptor-1 and correlates with the malignant phenotype of human melanoma. J Biol Chem 2003; 178: 46632–42.
25. Hejna M, Radere M, Zielinski CC. Inhibition of metastases by anticoagulants. J Natl Cancer Inst 1999; 91: 22–36.
26. Koenig A, Norgard-Sumnicht K, Linhardt R et al. Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. J Clin Invest 1998; 101: 877–89.
27. Wang L, Brown JR, Varki A et al. Heparin's anti-inflammatory effects require glucosamine 6-*O*-sulfation and are mediated by blockade of L- and P-selectins. J Clin Invest 2002; 110: 127–36.
28. Sasisekharan R, Shriver Z, Venkataraman G et al. Roles of heparan-sulphate glycosaminoglycans in cancer. Nat Rev Cancer 2002; 2: 521–8.
29. Ma A-Q, Geng J-G. Heparan sulfate-like proteoglycans mediate adhesion of human malignant melanoma A375 cells to P-selectin under flow. J Immunol 2000; 165: 558–65.
30. Döme B, Somlai B, Ladányi A et al. Expression of CD44v3 splice variant is associated with the visceral metastatic phenotype of human melanoma. Virchows Arch 2001; 439: 628–35.
31. Tímár J, Lapis K, Dudás J et al. Proteoglycans and tumor progression: Janus-faced molecules with contradictory functions in cancer. Sem Cancer Biol 2002; 12: 173–86.
32. Tímár J, Diczházi CS, Bartha I et al. Modulation of heparan-sulphate/chondroitin-sulphate ratio by glycosaminoglycan biosynthesis inhibitors affects liver metastatic potential of tumor cells. Int J Cancer 1995; 62: 755–61.
33. Fazekas K, Rásó E, Zarándi M et al. Basic HGF-like peptides inhibit generation of liver metastases in murine and human tumor models. Anticancer Res 2002; 22: 2575–80.
34. Da Silva MS, Horton JA, Wijelath JM et al. Heparin modulates integrin-mediated cellular adhesion: Specificity of interactions with alpha and beta integrin subunits. Cell Commun Adhes 2003; 10: 59–67.
35. Trikha M, Tímár J, Zacharek A et al. Role for $\beta 3$ integrins in human melanoma growth and survival. Int J Cancer 2002; 101: 156–67.
36. Trikha M, Zhou Z, Tímár J et al. Multiple roles for platelet gpIIb/IIIa and $\alpha v\beta 3$ integrins in tumor growth, angiogenesis and metastasis. Cancer Res 2002; 62: 2824–833.
37. Friedl P, Wolf K. Cell–cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer 2003; 3: 362–74.
38. Wolf K, Müller R, Borgmann S et al. Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. Blood 2003; 102: 3262–9.
39. Thodiyil P, Kakkar AK. Can low-molecular-weight heparins improve outcome in patients with cancer?. Cancer Treat Rev 2002; 28: 151–5.