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Mechanisms of heparin induced anticancer activity in experimental cancer models

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Abstract

Background
Retrospective analyses of clinical trials and prospective clinical studies have suggested that heparins may have an effect on cancer survival. This putative anticancer activity of heparins is supported by data from studies in animal tumor models.

Objective
To clarify the various potential mechanisms of heparin anticancer activity we evaluated the data from pre-clinical studies in which heparins have been tested as anticancer therapy.

Methods
Pre-clinical studies, published between 1960 and 2005 were assessed. Data was collected on the type and dose of heparin used, duration of exposure to heparin, interval between heparin administration and cancer cell inoculation, and the animal tumor model used. In addition, a distinction was made in the analysis between heparin effects on the primary tumor or on established metastases and effects on the metastatic potential of infused cells.

Results
Heparins seemed to affect the formation of metastasis rather than the growth of primary tumors. Chemically modified heparins with no or limited anticoagulant activity also showed anti-metastatic properties. Possible mechanisms to explain the effects on the process of metastases include inhibition of blood coagulation, inhibition of cancer cell-platelet and -endothelial interactions by selectin inhibition and inhibition of cell invasion and angiogenesis.

Conclusions
The anticancer activity of heparins depends more on inhibition of metastasis formation than on the effects on primary tumor growth. These effects are probably related to both coagulation and non-coagulation dependent factors. For a definitive proof of the anticancer activity of heparins in the clinic, prospective randomized trials especially in patients with early metastatic disease or in the adjuvant setting are urgently needed.
Introduction

Although the association of thrombosis and cancer is a well-established phenomenon, the role of activation of the coagulation cascade in cancer prognosis is not well defined. A critical role for activated coagulation factors in cancer biology has been suggested for years, and is supported by clinical studies with heparins and in preclinical studies in vivo and in vitro tumor models.

In a subgroup analysis of a study on venous thromboembolism (VTE) therapy, Prandoni et al observed a significant reduction in the three months mortality rate in the subgroup of cancer patients who received low molecular weight heparin (LMWH), nadroparin. Forty-four percent (8 of 18) of the cancer patients who were treated with unfractionated heparin (UFH) died during the study period as compared with only 7% (1 of 15) of those patients in the LMWH group (p=0.021).

The LMWH effect on survival was confirmed in a subsequent systematic review of nine studies on VTE-treatment, which showed a 49% reduction of the 3 months mortality (hazard ratio, HR 0.61:95%CI, 0.40-0.93) with LMWH relative to UFH.

The reduction in mortality was observed for various types of cancer, it was not the result of differences in fatal VTE or bleeding events and was unaltered after adjustment for other prognostic variables.

Prospective randomized trials have evaluated the survival effects of UFH or LMWH in patients with cancer. Tempelhoff et al studied the survival effect of prophylactic-dose LMWH versus UFH given for seven days postoperatively. In a group of 324 patients undergoing surgery for breast or pelvic cancer, the mortality rate in LMWH treated patients was 5.7% compared to 15.6% in UFH recipients (p=0.005).

In a trial of Lee et al 676 cancer patients with VTE were randomized to receive 6 months of dalteparin or Vitamin K antagonists (VKA). No difference in the one year mortality was detected. However, a post-hoc subgroup analysis suggested that in patients without metastatic disease the 1 year mortality was significantly reduced by dalteparin group (20%) relative to the VKA group (35%) (HR 0.50; 95% CI 0.27-0.95) (p=0.03). This difference was apparently not attributed to a difference in fatal thrombosis.

Two recent studies (FAMOUS and MALT) have evaluated the effects of LMWH on survival in cancer patients without VTE. In the FAMOUS study, 385 cancer patients without thrombosis were randomized between low-dose dalteparin (5000 IU/day) or placebo for 1 year. No significant difference in survival between the two study groups was detected. In the subgroup of patients with a relatively good prognosis at entrance in the study, dalteparin was associated with a better median survival (24 months versus 43 months; p=0.03). In the MALT study, 302 cancer patients without thrombosis were randomized to 6 weeks of nadroparin (2 weeks full therapeutic followed by 4 weeks half this dose) or placebo. The median survival was 6.5 months in the placebo group and 8.0 months in the nadroparin group (HR for mortality 0.75; 95% CI; 0.59-0.96).
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the a priori specified subgroup of patients with a life expectancy of at least 6 months, the median survival increased from 9.4 to 15.4 months (HR for mortality 0.64; 95% CI; 0.45-0.90).

Two trials studied the effect of UFH or LMWH in patients with small cell lung cancer. In both these studies a statistically significant survival advantage was observed in patients randomized to chemotherapy plus UFH or LMWH relative to patients who received chemotherapy alone.

Although several clinical studies strongly support an anticancer activity of heparins, and especially LMWH, many questions remain unresolved. The precise mechanism(s) by which LMWHs exerts their anticancer activity even after a short-term LMWH administration for example, remains unknown. It is also unclear, whether the observed anticancer effect of LMWHs is solely accounted for by their anticoagulant activity or if an influence on other biological processes is also involved. In this study we systematically reviewed the available evidence on the relationship between heparins and cancer from experimental studies in animal models.

Heparins

Heparins and low molecular weight heparins

Heparin is a highly sulphated member of the glycosaminoglycan (GAG) family. These molecules are long and unbranched disaccharide repeats and are located primarily in the cell membrane and in the extracellular matrix (ECM). Other members of the family of GAGs with known physiological significance are hyaluronic acid, dermatan sulphate, chondroitin sulphate and keratan sulphate. Heparin is abundant in granules of mast cells that line blood vessels and are present in mucosal tissue. The release of heparin from these granules in response to injury and its subsequent entry into the bloodstream leads to inhibition of blood clotting.

UFH, used in the clinic, are naturally-occurring glycosaminogycans from porcine or bovine origin with molecular weights in the range of 12,000-14,000 Daltons. LMWHs are derived from these UFH by chemical or enzymatic depolymerization, yielding fragments of approximately one third of the size of heparin (MW approximately 5000). The different types of LMWHs are prepared by different methods of degradation, and although they differ from each other to some extent in pharmacokinetic properties and anticoagulant profile, they are recognized to be quite comparable in clinical activity.

The antithrombotic activity of heparin is mediated by interaction with antithrombin (AT). Heparin molecules bind AT via a unique pentasaccharide motif that induces a conformational change in AT that enhances its antithrombotic activity manifold. The heparin-AT complex binds activated factor X via direct binding of AT thereby inactivating factor Xa. This anti Xa activity is equivalent between UFH and LMWHs.

The difference between UFH and LMWH is in their relative inhibitory activity against
factor Xa and factor IIa\(^{13}\). In contrast, the inactivation of factor IIa is mediated by the formation of a ternary complex consisting of heparin, AT, and factor IIa\(^{14}\). An 18-saccharide sequence in the heparin molecule is required to form such a trimolecular complex. The majority of LMWH chains are too short to form this complex. Thus LMWH have relatively little anti-IIa activity compared to UFH\(^{13}\).

Besides binding to antithrombin, UFH and LMWH can bind to a wide range of other proteins and molecules via electrostatic interactions with the polyanionic groups of the glycosaminoglycan chains\(^{16}\). These interactions are mediated by physicochemical properties of heparin polymers such as sequence composition, sulfation pattern, charge distribution, overall charge density and molecular size. As a consequence, UFH and LMWH have a wide variety of biological activities other than their anticoagulant effects.

**Modified heparins**

Several chemically modified heparins with a decreased anticoagulant activity have been synthesized to minimise the anticoagulant activity and to enhance the inhibitory effects of heparin on the tumor growth and metastasis. Periodate-oxidised (IO\(_4\) - heparin) and periodate-oxidised, alkaline-degraded LMWH (IO\(_4\) - LMWH)\(^{17}\), for instance, do not have a specific pentasaccharide structure to interact with antithrombin III and show a much lower anti-coagulant activity than UFH\(^{18}\). Other heparin derivatives have been produced which reduced the anticoagulant activity, like; N-acetylated, N-desulfated, O-desulfated or carboxyl-reduced heparin\(^{19,20}\). Also treatment of heparin with sodium borohydrate markedly reduced the anticoagulant activity of heparin\(^{21}\). Other modified heparins such as N-succinylated heparins (Succ 100 H) and N-succinylated LMWH (Succ 100-LMW-H) and very low molecular weight heparin (VLMWH) have been synthesised and showed to have also a low anticoagulant effect\(^{22}\). Same as the anticoagulant activity of a series of semisynthetic sulfaminoheparosan sulfates (SAHSs) with different degrees and distributions of 2-O or 3-O sulfation at glucosamine residues obtained by chemical modification of the *E coli* K4 polysaccharide\(^{23}\).

**Effects of heparins on experimental primary tumor growth and metastasis**

The effects of heparins on cancer have been studied in several animal models. Important variation in study design and type of tumor makes interpretation and comparison difficult. Variations include the type of heparin, heparin dose and duration of exposure, interval between heparin administration and cancer cell inoculation and the animal model used. In this review we discriminated between effects on the primary tumor or established metastases and effects on the metastatic potential of infused cells.
**Chapter 2**

**Effect of heparins on the outgrowth of primary tumors or established metastases**

Seventeen studies have evaluated the effect of heparins on subcutaneous implanted or chemically induced tumors in animals (Table 1). In only 4 of these studies heparin treatment induced a significant reduction of the primary tumor volume. The effect of heparins was not affected by the different methods of tumor induction in the various animal models. In a similar way, the interval between the start of heparin treatment and tumor induction was not a determinant factor for tumor growth inhibition. Other potential factors influencing the effect of heparins were the dose, type of heparins used and the duration of treatment. High doses of heparin (100 U 3 times for 15 days) seemed to be effective, although results have not been always concordant. Low doses of heparin or less than 15 days of administration did not affect tumor growth. Moreover, a very high dose of heparin given only three times in a week was not apparently capable to affect the primary tumor growth. Therefore it appears that long-term heparin administration at high concentration levels is necessary for an effect on the primary tumor growth.

In 2 studies a tumor growth inhibition was observed with chemically modified heparins (2,3-O-desulphated heparin, IO4-LMWH and NAC-HCPS) with low anticoagulant activity. That this is not the case for all modified heparins was demonstrated in the study of Sciumbata were Succ 100-LMWH and VLMH had no effect on tumor growth. In summary, the available data suggests that only high dosages of heparin and some chemically modified heparins may inhibit the primary tumor growth in various tumor models. In this regard the importance of a high dose of heparin is supported by studies in intra-peritoneal tumor models (Table 2). Heparin administration into the peritoneal cavity reduced the peritoneal tumor deposits in 6 out of 7 studies. This would suggest that high heparin concentrations around the tumor cells may be necessary. Because modified heparins with limited or no anticoagulant activity demonstrate anti-tumor activity suggests that the anti-tumor is not only dependent on the inhibition of coagulation.

**Effects of heparins on the development of metastases**

Metastases, rather than primary tumors, are responsible for most cancer deaths. Therefore it is worth speculating that heparins derive part or all of its effect on prognosis of cancer patients by interfering with haematogenous metastasis formation. This issue has been studied using both primary and secondary metastasis models. In the primary models, tumor cells are directly injected into the bloodstream and the resulting metastasis are recorded. In the secondary models, tumor cells are placed either subcutaneously or intraperitoneally followed by evaluation of the number and/or size of metastases developing in the lung. Table 3 summarizes data from 17 studies (1985-2005) investigating the effects of heparin on primary metastases (visceral metastases following intravenous cancer cell administration) and secondary metastases (visceral metastases following subcutaneous cancer cell inoculation). In most of these studies, 14 out of these 17 studies, heparin induced a significant reduction in the number of metastases.
Table 1. Effect of heparins on the outgrowth of primary tumors and established metastases

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cancer type</th>
<th>Animal</th>
<th>Cancer development</th>
<th>Sacrificed (days)</th>
<th>Interval</th>
<th>Doses schedule</th>
<th>Results</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[30]</td>
<td>Sarcoma T-241</td>
<td>C57BL/6J mice</td>
<td>Leg muscle</td>
<td>18</td>
<td>−2 h</td>
<td>1 dd sc</td>
<td>No effect</td>
<td>Heparin 50–200 U</td>
</tr>
<tr>
<td></td>
<td>Sarcoma DBA49</td>
<td>DBA mice</td>
<td>Bearing tumor in leg for several years</td>
<td>21</td>
<td>−2 h</td>
<td>1 dd sc</td>
<td>No effect</td>
<td>Heparin 50–200 U</td>
</tr>
<tr>
<td>[31]</td>
<td>Sarcoma MCG1-SS</td>
<td>CBA mice</td>
<td>s.c.</td>
<td>10</td>
<td>+48 h</td>
<td>6 days 3 dd s.c.</td>
<td>No effect</td>
<td>Heparin not further specified</td>
</tr>
<tr>
<td>[32]</td>
<td>Melanoma B16</td>
<td>C57BL/6J mice</td>
<td>s.c.</td>
<td>5–16</td>
<td>+48 h</td>
<td>6 days 3 dd s.c.</td>
<td>No effect</td>
<td>Heparin not further specified</td>
</tr>
<tr>
<td></td>
<td>Sarcoma MCG1-AS</td>
<td>CBA mice</td>
<td>s.c.</td>
<td>5–14</td>
<td>+48 h</td>
<td>6 days 3 dd s.c.</td>
<td>No effect</td>
<td>Heparin not further specified</td>
</tr>
<tr>
<td>[24]</td>
<td>Sarcoma MSLS</td>
<td>Holtzman rats</td>
<td>s.c.</td>
<td>8–15</td>
<td>+24 h</td>
<td>15 days 3 dd i.p.</td>
<td>Tumor growth Heparin 100 U reduced by 44%</td>
<td></td>
</tr>
<tr>
<td>[27]</td>
<td>PA-III cells 1 × 105 cells</td>
<td>Rats</td>
<td>Injected s.c.</td>
<td>42</td>
<td>+72 h</td>
<td>3 days/week i.v.</td>
<td>No effect</td>
<td>Heparin 200 U</td>
</tr>
<tr>
<td>[26]</td>
<td>Walker 256 carcino sarcoma</td>
<td>Sprague–Dawley</td>
<td>Injected s.c.</td>
<td>50</td>
<td>−48 h; 0; +48 h</td>
<td>7 days i.v.; 14 days i.v.; continuous</td>
<td>No effect</td>
<td>Heparin 600 U</td>
</tr>
<tr>
<td>[33]</td>
<td>Nb-Pr-A I-II</td>
<td>Rats</td>
<td>s.c.</td>
<td>21</td>
<td>Therapy tumor 90 mm3</td>
<td>1 dd i.v.</td>
<td>No effect</td>
<td>Heparin 15 U; Heparin 150 U</td>
</tr>
<tr>
<td>[34]</td>
<td>Nb-Pr-A I-II</td>
<td>Rats</td>
<td>s.c.</td>
<td>30</td>
<td>Therapy tumor 90 mm3</td>
<td>1 dd i.p.</td>
<td>No effect</td>
<td>Heparin 15 U</td>
</tr>
<tr>
<td></td>
<td>Nb-Pr-A I-III</td>
<td>Rats</td>
<td>s.c.</td>
<td>30</td>
<td>0</td>
<td>4 days/3 weeks i.p.</td>
<td>No effect</td>
<td>Heparin 150 U</td>
</tr>
<tr>
<td>[35]</td>
<td>Sarcoma NFSA 6 × 105 cells</td>
<td>C3 Hf/kam mice</td>
<td>Injected s.c.</td>
<td>23</td>
<td>+7 days</td>
<td>17 days 2 dd s.c. or orally</td>
<td>No effect</td>
<td>Heparin 15.7 U; Heparin not further specified</td>
</tr>
<tr>
<td></td>
<td>Mammary carcinoma MCA-K 6 × 105 cells</td>
<td>C3 Hf/kam mice</td>
<td>Injected s.c.</td>
<td>24</td>
<td>+10 days</td>
<td>14 days 2 dd s.c. or orally</td>
<td>No effect</td>
<td>Heparin 15.7 U; Heparin not further specified</td>
</tr>
<tr>
<td>[36]</td>
<td>Mammary carcinoma 3 × 104 cells</td>
<td>BR 6/Icrf mice</td>
<td>Injected s.c.</td>
<td>28</td>
<td>−24 h, 1 h</td>
<td>6 times/week i.p.</td>
<td>No effect</td>
<td>Heparin 40 U</td>
</tr>
<tr>
<td>[25]</td>
<td>Squamous carcinoma</td>
<td>Ddy mice</td>
<td>Induced</td>
<td>63</td>
<td>Therapy tumor 5 mm3</td>
<td>2 dd i.p.</td>
<td>Tumor growth Heparin 12.5 U reduced</td>
<td></td>
</tr>
</tbody>
</table>
Table 1. Effect of heparins on the outgrowth of primary tumors and established metastases

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cancer type</th>
<th>Animal</th>
<th>Cancer development</th>
<th>Sacrificed (days)</th>
<th>Interval</th>
<th>Doses schedule</th>
<th>Results</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[37]</td>
<td>Colon adeno carcinoma</td>
<td>SW480 3 × 10⁶ cells</td>
<td>Balb-c mice Injected s.c.</td>
<td>84</td>
<td>−24 h</td>
<td>2 dd s.c.</td>
<td>No effect</td>
<td>Heparin 3 U</td>
</tr>
<tr>
<td>[22]</td>
<td>B16-BL melanoma cells 2 × 10⁵ cells</td>
<td>C57BL/6 mice Injected s.c.</td>
<td>20</td>
<td>−10 min</td>
<td>2 times/week s.c.</td>
<td>No effect</td>
<td>Chemically modified heparin</td>
<td></td>
</tr>
<tr>
<td>[28]</td>
<td>CaPan-2 3 × 10⁶ cells</td>
<td>Balb/c mice Injected s.c.</td>
<td>35</td>
<td>+24 h</td>
<td>1 dd s.c.</td>
<td>Tumor growth reduced</td>
<td>Chemically modified heparin</td>
<td></td>
</tr>
<tr>
<td>[38]</td>
<td>Colon adeno carcinoma</td>
<td>WAG-Rij rats Injected i.v. portal vein</td>
<td>24</td>
<td>−1 h</td>
<td>1 dd i.p.</td>
<td>No effect</td>
<td>LMWH; not further specified</td>
<td></td>
</tr>
<tr>
<td>[29]</td>
<td>Lewis lung cancer, 3LL 1 × 10⁷ cells</td>
<td>C57BL/6 mice Injected s.c.</td>
<td>20</td>
<td>Tumor 100–200 mm³ (Day 14)</td>
<td>Days 14–20 1 dd s.c.</td>
<td>Tumor growth reduced</td>
<td>Chemically modified heparin</td>
<td></td>
</tr>
<tr>
<td>[29]</td>
<td>B16 melanoma 1 × 10⁷ cells</td>
<td>C57BL/6 mice Injected s.c.</td>
<td>20</td>
<td>Tumor 100–200 mm³ (Day 14)</td>
<td>Days 14–20 1 dd s.c.</td>
<td>Tumor growth reduced</td>
<td>Chemically modified heparin</td>
<td></td>
</tr>
<tr>
<td>[39]</td>
<td>Lewis lung cancer, 1 × 10⁶ cells</td>
<td>C57/BL6 Injected s.c.</td>
<td>22</td>
<td>+6 days</td>
<td>Days 6–22 every 3 days s.c.</td>
<td>No effect</td>
<td>Heparin 5 U; LMWH 5.7 U anti Xa</td>
<td></td>
</tr>
</tbody>
</table>

Interval refers to the interval between cancer cell administration and start of heparin treatment; MCG1-SS, Syngenic 20MC (methylcholanthrene) induced rhabdomyosarcoma; MCG1-AS, rhabdomyosarcoma, a solid ascites form of MCG1-SS; MSLS, Murphy-Sturm Lymphosarcoma; PA-III cells, Rat prostate adenocarcinoma cells; 3LL, Lewis lung cancer cells; CaPan-2, human pancreatic adenocarcinoma; Nb-Pr-A.I-II, Nb rat prostatic adenocarcinoma; NFSA, a fibrosarcoma; MCA-K, mammary carcinoma.
Table 2. Effect on intraperitoneal tumor growth after treatment with heparins in a laparoscopic model

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cancer type</th>
<th>Animal</th>
<th>Sacrificed (days)</th>
<th>Interval</th>
<th>Doses schedule</th>
<th>Results</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[40]</td>
<td>MBT-2 cells $1 \times 10^5$ cells</td>
<td>C3H mice</td>
<td>16</td>
<td>0</td>
<td>Single i.p.</td>
<td>Reduced tumor weight</td>
<td>Heparin 10 U</td>
</tr>
<tr>
<td>[41]</td>
<td>DHD/K12/TRb $1 \times 10^4$ cells</td>
<td>BD IX rats</td>
<td>28</td>
<td>0</td>
<td>Single i.p.</td>
<td>Reduced tumor weight</td>
<td>Heparin 20 U</td>
</tr>
<tr>
<td>[42]</td>
<td>DHD/K12/TRb $1 \times 10^4$ cells</td>
<td>DB IX rats</td>
<td>28</td>
<td>0</td>
<td>Single i.p.</td>
<td>Reduced tumor weight</td>
<td>Heparin 20 U</td>
</tr>
<tr>
<td>[43]</td>
<td>Mammary adenocarcinoma $2 \times 10^7$ cells</td>
<td>Dark Agouti rats</td>
<td>7</td>
<td>+4 min</td>
<td>Single i.p.</td>
<td>Reduced tumor weight</td>
<td>Heparin 200 U</td>
</tr>
<tr>
<td>[44]</td>
<td>DHD/K12/TRb $1 \times 10^6$ cells</td>
<td>BD IX rats</td>
<td>28</td>
<td>0</td>
<td>Single i.p.</td>
<td>No effect</td>
<td>Heparin 5 U</td>
</tr>
<tr>
<td>[45]</td>
<td>Colon carcinoma CC531 $5 \times 10^6$ cells/ml</td>
<td>WAG rats</td>
<td>21</td>
<td>0</td>
<td>Single i.p. 1 dd s.c.</td>
<td>Reduced tumor weight</td>
<td>LMWH; not further specified</td>
</tr>
<tr>
<td>[46]</td>
<td>CC 531 Colon carcinoma $5 \times 10^6$ cells/ml</td>
<td>WAG rats</td>
<td>21</td>
<td>0</td>
<td>Single i.p. 1 dd s.c.</td>
<td>Reduced tumor weight</td>
<td>LMWH; not further specified</td>
</tr>
</tbody>
</table>

MBT-2, murine bladder tumor cells; DHD/K12/TRb (ECACC), colonic adenocarcinoma of the rat.

\(^a\) A significant decrease in tumor weight was observed in a group of rats that received heparin together with the group that received 2 ml fresh blood plus 200 U heparin compared with controls.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Cancer type</th>
<th>Animal</th>
<th>Site of metastases</th>
<th>Sacrifice (days)</th>
<th>Interval</th>
<th>Doses schedule</th>
<th>Results</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[34]</td>
<td>Nb-Pr-AI-II s.c.</td>
<td>Rats</td>
<td>Lung</td>
<td>30</td>
<td>Therapy start tumor 30 mm3</td>
<td>30 days 1 dd i.p.</td>
<td>Reduced spontaneous metastasis</td>
<td>Heparin 17.5 U</td>
</tr>
<tr>
<td></td>
<td>Nb-Pr-AI-III s.c.</td>
<td>Rats</td>
<td>Lung</td>
<td>30</td>
<td>0</td>
<td>4 times/week during 3 weeks i.p.</td>
<td>Reduced spontaneous metastasis</td>
<td>Heparin 175 U</td>
</tr>
<tr>
<td>[35]</td>
<td>NFSA 2 × 105 cells i.v.</td>
<td>C3 Hf/Kam mice</td>
<td>Lung</td>
<td>13</td>
<td>+4 days</td>
<td>9 days 2 dd s.c.</td>
<td>Reduced metastasis</td>
<td>Heparin 15.6 U</td>
</tr>
<tr>
<td></td>
<td>MCA-K 3 × 105 cells i.v.</td>
<td>C3 Hf/Kam mice</td>
<td>Lung</td>
<td>27</td>
<td>+7 days</td>
<td>14 days 2 dd s.c.</td>
<td>Reduced metastasis</td>
<td>Heparin 15.6 U</td>
</tr>
<tr>
<td>[19]</td>
<td>B16–BL6 melanoma cells</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>20</td>
<td>0</td>
<td>Cancer cells incubated with heparins for 2 h</td>
<td>Reduced metastasis</td>
<td>Heparin not further specified; chemically modified heparins</td>
</tr>
<tr>
<td>[50]</td>
<td>Sarcoma L-1 5 × 104 cells i.v.</td>
<td>Balb/c mice</td>
<td>Lung</td>
<td>14</td>
<td>−1 h</td>
<td>3 days 2 dd s.c.</td>
<td>Moderately reduced metastasis</td>
<td>Heparin 21 U</td>
</tr>
<tr>
<td></td>
<td>Sarcoma L-1 1 × 106 cells i.v.</td>
<td>Balb/c mice</td>
<td>Lung</td>
<td>14</td>
<td>−1 h</td>
<td>3 days 2 dd s.c.</td>
<td>Moderately reduced metastasis</td>
<td>Heparin 21 U</td>
</tr>
<tr>
<td>[51]</td>
<td>BR 6 mammary 3 × 104 cells i.v.</td>
<td>BR 6/Icrf mice</td>
<td>Lung</td>
<td>18</td>
<td>−24 h and −1 h</td>
<td>Single i.p.</td>
<td>Reduced metastasis</td>
<td>Heparin 40 U</td>
</tr>
<tr>
<td></td>
<td>BR 6 mammary 3 × 104 cells s.c.</td>
<td>BR 6/Icrf mice</td>
<td>Lung</td>
<td>26</td>
<td>−24 h and −1 h</td>
<td>4 days 3 dd i.p.</td>
<td>Reduced spontaneous metastasis</td>
<td>Heparin 40 U</td>
</tr>
<tr>
<td></td>
<td>Mammary carcinoma 3 × 104 cells s.c.</td>
<td>BR 6/Icrf mice</td>
<td>Lung</td>
<td>21–28</td>
<td>0</td>
<td>6 times/week i.p.</td>
<td>Reduced spontaneous metastasis</td>
<td>Heparin 40 U</td>
</tr>
<tr>
<td>[48]</td>
<td>DHD/K12/PRO 2 × 107 cells i.v.</td>
<td>BDIX rats</td>
<td>Liver</td>
<td>90</td>
<td>0</td>
<td>7 days i.v. Continuous</td>
<td>Reduced metastasis</td>
<td>NS Heparin 100 U</td>
</tr>
<tr>
<td>[22]</td>
<td>B16–BL6 melanoma 1 × 105 cells i.v.</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>21</td>
<td>−10 min</td>
<td>Single s.c.</td>
<td>Reduced metastasis</td>
<td>Chemically modified heparin</td>
</tr>
<tr>
<td>[52]</td>
<td>B16–BL6 melanoma 1 × 105 cells i.v.</td>
<td>C57/BL6 mice</td>
<td>Lung</td>
<td>15</td>
<td>−20 min</td>
<td>Single i.p.</td>
<td>Reduced metastases</td>
<td>LMWH 13 U</td>
</tr>
<tr>
<td></td>
<td>13762 MAT Mammary 2 × 105 cells i.v.</td>
<td>Fisher 344 rats</td>
<td>Lung</td>
<td>15</td>
<td>−20 min</td>
<td>Single i.p.</td>
<td>Reduced metastasis</td>
<td>LMWH 468 U</td>
</tr>
<tr>
<td>Reference</td>
<td>Cancer type</td>
<td>Animal</td>
<td>Site of metastases</td>
<td>Sacrifice (days)</td>
<td>Interval</td>
<td>Doses schedule</td>
<td>Results</td>
<td>Drugs</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td>[38]</td>
<td>CC531 Colon carcinoma 1 × 10⁵ cells i.v.</td>
<td>WAG-Rij rats</td>
<td>Liver</td>
<td>8</td>
<td>−1 h</td>
<td>LMWH 8 days 1 dd i.p.; UFH 8 days 2 dd i.p.</td>
<td>No effect</td>
<td>LMWH/heparin 2.0 mg/kg</td>
</tr>
<tr>
<td>[53]</td>
<td>LS180 Colon carcinoma 3–4 × 10⁵ cells i.v.</td>
<td>Littermate RAG2 mice</td>
<td>Lung</td>
<td>42</td>
<td>−30 min</td>
<td>Single i.v.</td>
<td>Reduced metastasis</td>
<td>Heparin 100 U</td>
</tr>
<tr>
<td>[29]</td>
<td>Lewis lung 3 × 10⁵ cells</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>14</td>
<td>?</td>
<td>7 days 1 dd i.v.</td>
<td>Reduced metastasis</td>
<td>Chemically modified heparin</td>
</tr>
<tr>
<td>[54]</td>
<td>B16 melanoma 3 × 10⁵ cells i.v.</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>14</td>
<td>0</td>
<td>Single i.v.</td>
<td>Reduced metastasis</td>
<td>Chemically modified heparin</td>
</tr>
<tr>
<td>[47]</td>
<td>B16 melanoma 2 × 10⁵ cells i.v.</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>15</td>
<td>−4 h</td>
<td>14 days 1 dd s.c.</td>
<td>Reduced metastasis</td>
<td>LMWH 10 mg/kg</td>
</tr>
<tr>
<td>[21]</td>
<td>H11 cells 1 × 10⁶ cells i.v.C57BL/6 mice</td>
<td>C57BL/6 mice</td>
<td>Lung</td>
<td>14</td>
<td>−10 min</td>
<td>Single s.c.</td>
<td>Reduced metastasis</td>
<td>Chemically modified heparin</td>
</tr>
<tr>
<td>[55]</td>
<td>MC38GFP 1 × 10⁵ cells</td>
<td>C57/BL6J mice</td>
<td>Lung</td>
<td>27</td>
<td>−30 min</td>
<td>Single s.c.</td>
<td>Reduced metastasis</td>
<td>UFH 6.56 U/19.68 U</td>
</tr>
</tbody>
</table>
Table 3. Effects of heparins on experimentally induced lung or liver metastases after intravenous injection of cancer cells

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cancer type</th>
<th>Animal</th>
<th>Site of metastases</th>
<th>Sacrifice (days)</th>
<th>Interval</th>
<th>Doses schedule</th>
<th>Results</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[56]</td>
<td>HT168-M1</td>
<td>BALB/c SCID mice</td>
<td>Lung</td>
<td>49</td>
<td>−7 h</td>
<td>3 days</td>
<td>Reduced metastasis</td>
<td>UFH 0.5–5 IU</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁶ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NFSA, fibrosarcoma; MCA-K, mammary carcinoma; Nb-Pr-A I-II, rat prostate adenocarcinoma androgen insensitive tumor type I; Nb-Pr-A I-III, rat prostate adenocarcinoma androgen insensitive tumor type III; H11 cell line established from Lewis lung 3LL-derived cell line; LL, Lewis Lung carcinoma cell line; Colon26, colon carcinoma cell line; B16F0, Melanoma cell line; FBJ, Osteosarcoma cell line; MC38GFP, mouse colon carcinoma cell line stable transfected with enhanced green fluorescent protein; HT168-M1, human melanoma cell line.
Heparin induced anticancer activity

The timing of heparin administration in relation to the intravenous inoculation of cancer cells is likely to be relevant, because intravascular exposure of cancer cells to heparins or heparin effects may affect the capacity of survival and endothelial adhesion of cancer cells, events necessary for successful metastases. Antimetastatic activity of heparins was showed in 10 studies in which heparin was administrated just before intravenously tumor cell inoculation. In 1 study, showing an inhibitory effect of heparin, heparin administration started at 4 or 7 days after cancer cell inoculation. In the study of Amirkhosravi et al the interval was 4 hours. In this study the LMWH, tinzaparin, resulted in an APTT of 200 seconds, 4 hours after administration, reflecting the presence of a significant anticoagulant effect at the time of intravenous cancer cell inoculation.

Three studies investigated the anti-tumor effects of heparins with reduced anticoagulant activity. These chemically-modified heparins, NAH-HCPS, LAC heparin and ITF 1164 were also able to inhibit tumor metastases, suggesting that the anti-metastatic effect was not dependent on anticoagulation. These chemically modified heparins had a pleiotropic action including anti-adhesion, anti ECM degradation, anti-platelet cancer complex formation and anti-angiogenesis activity, indicating the multipotency of these compounds in inhibition of almost all steps involved in the later phases of haematogenous metastasis development.

In 2 of the 3 studies which found no antimetastatic capacity of heparin the cancer cells were directly injected into the portal vein to induce liver metastases. The inoculation model may not be the ideal experimental set-up to investigate the anti-metastatic effects of heparins. The formation of metastases in the liver after administration of cancer cells may have involved mechanical entrapment of the cancer cells in the sinusoids due to their large size and their limited plasticity as was demonstrated by Mook et al using the same animal model and the same cancer cells. Moreover Mook et al demonstrated that these interactions between cancer cells and endothelial cells did not occur because endothelial cells retracted rapidly and the interactions occurred between cancer cells and hepatocytes. Therefore, in the model of Smorenburg et al and Nagawa et al, the molecular interactions that are sensitive to heparin may not have occurred.

In the third negative study development of metastases was decreased in the heparin group, but this effect was not significant. Also in this study the heparin dose was rather low.

**Mechanisms of heparin induced anticancer activity**

The reviewed studies in experimental models indicate that heparins are able to inhibit cancer metastasis more than tumor growth. Haematogenous metastasis is a complex and highly regulated process in which cancer cells detach from the primary tumor, migrate across blood vessel walls into the bloodstream, disperse throughout the body, adhere to endothelial cells and penetrate into surrounding tissues. During the
intravascular phase, cancer cells are extremely vulnerable to a variety of host defense mechanisms and only a few of the cancer cells that enter the bloodstream result in manifest metastases leading to fatal outcome. However, most of the animal studies on experimental metastasis involve the intravenous injection of tumor cell in the tail vein, resulting in pulmonary tumor foci. Over 90% of the tumor cells become entrapped in the vasculature of the lungs immediately after tail vein injection\textsuperscript{57}. This entrapment occurs regardless of anticoagulation or fibrinogen deficiency and is immediately followed by tumor cell induced coagulation and intravascular fibrin deposition. Tissue factor (TF), often highly expressed on tumor cells, plays an important role in the local activation of the coagulation cascade and the formation of the tumor-fibrin clot. The rapid decline of platelet counts following intravascular tumor cell inoculation is probably the result of platelet sequestration in the tumor-fibrin clot\textsuperscript{58}. Tumor cell induced coagulation and platelet activation may prolong the survival of tumor cells in the lung vessels. In these fibrin platelet complexes tumor cells may growth or die intravascular, or adhere to the endothelial wall followed by invasion into the lung parenchyma. The last process is also facilitated by the local generation of thrombin, thrombin induced platelet activation, and the local release from platelets of angiogenesis (vascular endothelial growth factor, VEGF) and proliferation (platelet factor 4, PF-4) inducing factors. Thrombin induced platelet activation results in activation of the platelet integrin receptor GP IIb/IIIa\textsuperscript{59}, allowing binding of platelets to fibrinogen, and induction of P-selectin from $\alpha$ granules, a process that is dependent on protease-activated receptor-4 (PAR-4) signaling. Increased levels of P-selectin on platelets and endothelial cells, contributes to the adherence of platelets and endothelial cells to aberrantly expressed carbohydrates on cancer cells\textsuperscript{60} (see next chapter). These data demonstrate that platelet activation and fibrin formation are both important mechanisms by which tumor cells promote haematogenous metastasis. Several experiments in animal models have demonstrated that a low platelet count or inhibition of platelet activation result in a decrease of the metastatic potential of tumor cells. In PAR-4 deficient mice and in Nf-E2 mice, mice lacking circulating platelets, haematogenous metastasis are significantly reduced\textsuperscript{61}. In addition, antagonists of GP\textsubscript{IIb/IIa}, Abciximab and XV45, are strong inhibitors of tumor cell platelet activation and the development of metastasis\textsuperscript{62-64}. The most challenging problem of the use of antiplatelet drugs in cancer is the lack of selectivity. Indeed, the currently available anti platelet drugs affect both haemostasis and cancer-induced platelet activation. The next part of this paper describes the potential mechanisms of heparins as anticancer drugs. Potential effects of heparins on the process of metastasis are; inhibitory effects of heparins on blood coagulation, inhibition of cancer cell-platelet and endothelium interactions during intravascular dissemination, inhibition of heparanase, and competition with growth factor binding to heparan sulphate proteoglycans (HSPGs).
Heparin induced anticancer activity

Coagulation as the target
The tumor associated activation of the coagulation cascade has been emended in both
the formation of tumor stroma and the promotion of metastasis. Most solid tumors
contain considerable amounts of fibrinogen-derived products suggesting that fibrin
is important in tumor stroma formation. Although inhibition of fibrin formation
by heparins is an attractive hypothesis for their anticancer effects, the evidence that
heparins directly affect tumor growth is limited. In addition to the role of coagulation-
induced fibrin in tumor stroma formation, coagulation factors may be involved in the
process of metastasis as well. Studies in fibrinogen-deficient mice demonstrated that fibrin(ogen) appears to facilitate metastasis by enhancing the sustained survival of
individual cell emboli in the vasculature. In the fibrinogen deficient animal model the
development of lung metastasis, upon intravenous cancer cell inoculation, was strongly
diminished compared to normal mice. In the same model the specific thrombin inhibitor
hirudin further diminished the metastatic potential of circulating cancer cells, suggesting
that thrombin facilitates metastasis partly in a fibrinogen-independent way. Heparins are
able to interfere with these coagulation dependent mechanisms of metastases. Heparins
have been shown to shorten the retention time of tumor cells in the lungs and reduce the
development of lung metastasis in this way. It also inhibits thrombin and fibrin formation
and by virtue of preventing thrombin generation and activity, can thus inhibit platelet
activation.

An important aspect in coagulation activation is the expression of tissue factor (TF) on
cancer cells.
The expression of TF is inhibited in a fXa-dependent manner by tissue factor pathway
inhibitor (TFPI). TFPI is mainly synthesized in and localized to vascular endothelial cells
and is able to inhibit metastatic tumor growth in mice also suggesting a role for thrombin
in the metastatic process. Because heparins can also cause the release of tissue factor
pathway inhibitor (TFPI), which reduces the pro-coagulant activity of cancer cells, it is
tempting to speculate that TFPI is one of the targets involved in the anticancer activity of
heparins.

Because several studies demonstrated that also non-antiocoagulant heparins have the
potential to inhibit metastasis, other, non anti-coagulant, effects of heparins may
be important as well.
For example, non-antiocoagulants can potentially cause the release of TFPI from the vascular
endothelium and TFPI may contribute to their anti metastatic effect partially independent
of the role in the coagulation cascade but as an inhibitor of angiogenesis.

Selectins as the target
Ample evidence from pre-clinical studies supports the concept that intravascular survival
and arrest of cancer cells on the endothelial surface is facilitated by interactions between
cancer cells and platelets. The formation of cancer cell-platelet complexes provides a shield that protects them from immune competent cells and favours cancer cell adhesion to vascular endothelium. This interaction between cancer cells and platelets and endothelial cells is facilitated by binding of glycoproteins in the plasma membrane of cancer cells to selectins on platelets and endothelium. Altered cell-surface glycosylation of glycans (mucins), which are high-molecular-weight molecules containing a protein core substituted with a large number of O-linked carbohydrates, is a prominent feature of cancer progression. Relatively few types of glycan alterations are associated with epithelial cancer. Sialyl Lewis\(^x\) and Lewis\(^a\) (sLex and sLe\(^a\)) epitopes are two of them. These sLe\(^a\) epitopes on carcinoma mucins are frequently associated with an advanced and further metastatic progression.

Selectins are adhesion receptors that recognize these altered carbohydrate structures. Their physiological function in mediating cell adhesion has been shown during inflammation, immune responses and wound repair. Selectins are primary responsible for the first steps in cell adhesion and in the absence of selectins, all subsequent steps mediated by integrins and other adhesion molecules are either substantially delayed or do not occur. Selectins are expressed on leukocytes (L-selectin), platelets (P-selectin) and the vascular endothelium (E- and P-selectin). Whereas L-selectin is constitutively expressed on neutrophils, monocytes, and naive lymphocytes, P-selectin is stored in secretory granules of resting platelets and endothelium, is rapidly translocated to the cell surface upon activation by thrombin and histamine. E-selectin is newly synthesized in endothelial cells via transcriptional activation initiated by various proinflammatory agonists such as IL-1, TNF-alpha and endotoxins. All three selectins can bind sialylated, fucosylated, or in some cases, sulfated glycans on glycoproteins, glycolipids, and proteoglycans. The tetrasaccharides sLe\(^x\) and sLe\(^a\) have been identified as the minimal ligands for all three types of selectins.

Heparin and heparin-like oligosaccharides can inhibit L-selectin and P-selectin binding to sLe\(^x\)-related compounds. Inhibition of L-selectin and P-selectin binding results in inhibition of the inflammatory response and may attenuate tumor metastasis.

**Heparanase as target**

A critical event in the process of cancer invasion and metastasis is the degradation of various components of the ECM, including collagen, laminin, fibronectin and HSPGs. Cancer cells are able to accomplish this task through the concerted actions of enzymes such as matrix metalloproteases, serine proteases, cysteine proteases and endoglycosidases. Among the endoglycosidases, heparanase, secreted by cancer cells, can destroy various ECM components favouring tumor invasion.

Heparanase expression is rare in normal tissue, but is evident in many human tumors where it significantly increases both the angiogenic and metastatic potential of cancer cells. Elevated heparanase expression in humans has been correlated with advanced...
Heparin induced anticancer activity

disease and metastasis of tumors of the breast, colon, ovary, bladder, pancreas, acute myeloid leukemia, non-small cell lung cancer and myelomas.

Heparanase is important for the cleavage of heparan sulphate (HS) groups from HSPGs. These HS groups are growth factor-bearing structures linked to the protein core of HSPGs and localized in the ECM and in plasma membranes of cells. These structures are involved in storage of growth factors, such as bFGF and VEGF and function as their co-factors in activation of plasma membrane receptors and downstream signaling. Heparanase does not completely digest HS chains but it cleaves the glycosidic bonds of HS chains at only a few sites, producing fragments which seems even more powerful than the native HS in potentiating the activity of bound growth factors.

Several reports showed that heparin and some chemically modified species of heparin as well, inhibit tumor cell heparanase activity and that the inhibition of heparanase activity correlates with a lower metastatic potential.

**Heparan sulphate proteoglycans as the target**

Soluble heparins compete with HS groups on HSPGs for the binding of growth factors and other proteins and may cause release of these proteins from the ECM. Both UFH and LMWH have been shown to inhibit bFGF-induced angiogenesis in a human in vitro angiogenesis model by disrupting the bFGF-HS interactions. In man, therapeutic doses of UFH can indeed cause an increase in plasma levels of growth factors, such as bFGF. In contrast, Soker et al showed that LMWH but not UFH can inhibit the binding of growth factors to their high affinity receptors as a result of its smaller size. In vitro, heparin fragments of less than 18 saccharide residues reduce the activity of VEGF and fragments of less than 10 saccharide residues inhibits the activity of bFGF.

Small heparin fractions have also been shown to inhibit VEGF and bFGF-mediated angiogenesis in vivo, in contrast to UFH. The relevance of HSPGs and growth factors as target for the heparin is very complicated and remains uncertain.

**Concluding remarks**

The process of tumor metastasis is a highly complicated, involving coagulation dependent and independent mechanism. Tumor cell induced platelet aggregation and activation plays an important role in the tumor cell protection and successful adherence to and invasion through the endothelial wall. Although some anti-platelet drugs exhibit potent anti-metastatic activity, this review focuses especially on the activities of heparins. Many experimental studies, reviewed here, support the hypothesis that heparin mainly affects the metastatic cascade rather than the primary tumor growth. In most experimental metastasis models tumor cells are injected intravenously in the tail vein resulting in pulmonary metastasis. During this process intravascular entrapment of tumor cells in the first capillary network (lungs) is followed by activation of coagulation via TF on the tumor
cells, platelet aggregation and activation. In this process thrombin plays an essential role, partially coagulation independent. Heparins are able to interfere at several sites in this sequence of events.

Because chemically modified heparins with no or limited anticoagulant activity also showed anti-metastatic properties in animal models, coagulation independent mechanisms may contribute to the anticancer activity of heparins as well\^[21, 22, 54, 110]. Heparin induced inhibition of selectin-mediated cell-cell interactions, heparanase and angiogenesis inhibition and stimulation of TFPI release could at least partly explain the non anticoagulant heparin anti-metastatic activity.

How to explain the activity of agents that mainly affect metastasis when clinical anti cancer effects of heparins have been demonstrated in patients who already have a metastatic disease? Increasing evidence emerges that the ongoing process of metastasis in patients with cancer contributes to cancer progression and further dissemination. Several studies demonstrated that circulating cancer cells are detectable in the circulation of most patients with cancer\^[111] and that the number of circulating cancer cells is correlated with prognosis\^[112]. These data suggest that in patients with metastatic disease interruption of the metastatic cascade with heparins may affect cancer outcome. This may also explain the phenomenon that heparins exert their anticancer activity mainly in patients with limited metastatic disease, whereas no effect is seen in patients with end stage disease\^[6-7].

In contrast to cytotoxic chemotherapy, where the target is the cancer cell itself, heparins mainly affect targets outside the cancer cell. The process of metastasis is probably not cancer-type specific and more attributable to the contribution of common extra cellular mechanisms. This explains that the favorable impact of heparins on cancer outcome is a general phenomenon and probably not restricted to specific cancer types.

It can be concluded that heparins have an anticancer effect, but still many issues remain to be unanswered: what type of heparin, what dose and what duration of administration are optimizing anticancer effects. However the main issue is a definite proof that heparins contribute to a better cancer outcome. This answer is urgently needed and necessitates the design of prospective randomized trials especially in patients with early metastatic disease or in the adjuvant setting.
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Chapter 2

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diminish its anticoagulant but preserve its heparanase-inhibitory, angiostatic, anti-tumor and anti-

heparin-carrying polystyrene (NAC-HCPS) affects angiogenesis and inhibits subcutaneous induced


Chapter 2


Chapter 2


Heparin induced anticancer activity


