The role of blood coagulation in cancer, inflammation and embryonic development
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Do anticoagulants affect colon cancer?

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(Submitted)
Abstract

Clinical trials have shown life-prolonging effects of low-molecular-weight heparins (LMWHs) in a wide variety of cancer patients. From animal studies it is known that the development of metastasis is dependent on the activation status of the blood coagulation cascade and that metastasis can be inhibited by anticoagulants. However, hypercoagulability due to a FVL disorder did not increase metastasis of colon cancer cells in the liver. This raises the question whether colon cancer cells are dependent on the coagulation cascade to metastasize and whether anticoagulants inhibit colon cancer metastasis.

We investigated the effects of LMWH on the development of metastasis in a mouse colon carcinoma model.

Mice received LMWH intraperitoneally by a micro-osmotic pump or were sham operated in an experimentally induced colon cancer metastasis model to generate long term therapeutic plasma levels of LMWH. Simultaneously, mice received MCA-38 syngeneic colon cancer cells in the portal vein. After 21 days, the mice were sacrificed and the livers were evaluated for the amount, size and morphology of the tumors.

LMWH anticoagulant activity as measured by anti-Xa plasma levels at 9 days after cancer cell injection was in the therapeutic range with average levels of 0.35 IU anti-Xa/ml. No difference in liver weight, number and size of metastases were found between LMWH-treated mice and controls. Differences in fibrin/fibrinogen content were not found between tumors of treated mice and tumors of controls.

Conclusion: LMWH does not affect colon cancer metastasis in mouse liver.
Introduction

Cancer patients often suffer from venous thromboembolic complications [1-3]. Consequently, cancer patients are often treated with anticoagulants including low-molecular-weight heparins (LMWHs). Meta-analysis of the literature indicated that treatment with heparins affects survival of cancer patients [4, 5]. Cancer patients with venous thromboembolism (VTE) that had been treated with LMWH showed improved survival over cancer patients with VTE treated with unfractionated heparin [6]. Prospective randomized clinical trials showed life-prolonging effects of LMWHs in cancer patients with and without thrombotic complications [7-9].

Various animal studies documented an inhibitory effect of anticoagulants on cancer cell metastasis [5,10,11]. Most studies investigated the effect of heparins on lung metastasis induced by B16 melanoma cancer cell administration into the tail vein [10, 12-15]. Several studies showed that different components of the coagulation system, such as tissue factor (TF) [16], factor Xa [17], thrombin [18] and fibrinogen [19] can contribute to tumor progression by promoting metastasis, whereas the TF pathway inhibitor (TFPI) inhibits metastatic tumor growth in mice [20]. All these results imply that coagulation facilitates metastasis and imply potential beneficial effects of anticoagulants in the prevention or treatment of cancer. Indeed, a pulmonary mouse model of hypercoagulability, based on the factor V Leiden (FVL) mutation, facilitates metastasis [21]. In contrast, a disorder of the FVIII gene -mice suffering from haemophilia- showed a protective effect on cancer cell metastasis [21]. All these data suggest that the development of metastasis is dependent on the activation status of the blood coagulation cascade.

However, the latter findings are in contrast with the findings of Klerk et al [22], who showed that in a colon carcinoma model -where hypercoagulability was also induced by a FVL mutation- no effect on tumor development was evident. FVL mice and control mice appeared to develop colon cancer metastasis in the liver to a similar extent, suggesting that this type of metastasis is not dependent on the activation status of the blood coagulation cascade. Consequently, anticoagulants might not be effective in the treatment of colon cancer.

In order to explore whether long term therapeutic levels of LMWH affect the development of colon cancer metastasis, we investigated the effect of LMWH administration using an intraperitoneally implanted micro-osmotic pump on the development of liver metastasis. We compared liver weight, number and size of macroscopically-visible tumor nodules on the liver surface, and fibrin/fibrinogen content of the tumors of LMWH-treated and sham operated mice.

Methods

Animals

Twenty C57 black/6 mice (Charles Rivers, Someren, The Netherlands) with body weight of 22-26 g were kept under constant environmental conditions with a 12 h dark/12 h light cycle and free access to food and water. Animals had been kept under these conditions for at
least two weeks before the start of experiments to acclimatize. All animal experiments were in agreement with the Animal Ethic Committees of the Academic Medical Center, University of Amsterdam.

**Tumor model**

For the model of colon cancer metastasis in mouse liver, cells of an established syngeneic mouse colon carcinoma cell line, MCA-38 (kindly provided by Dr. E. Nelson, National Cancer Institute, Frederick MD, USA), were cultured, harvested and single cell suspensions were prepared for inoculation [23]. Each group consisted of 10 mice. Mice were randomly assigned to have a mini-osmotic pump implanted or were sham operated. The animal model that was used has previously been described by Griffini and colleagues [24].

Briefly, a small midline incision was made in the abdominal wall of the mice under anaesthesia with FFM mix (1 ml Hypnorm, 1 ml Midazolam and 2 ml water, 0.07 ml/10 g body weight, intraperitoneally administrated). A suspension containing 3.6x10^5 cells in 100 μl PBS was injected into the portal vein with a 30-gauge needle. In order to prevent peritoneal seeding, the portal vein was closed by applying pressure with a piece of spongostan (Medical Workshop, Groningen, The Netherlands) until bleeding had stopped. In mice allocated to the LMWH treatment group, the osmotic pump was inserted into the abdomen. The abdomen was closed using stitches (Perma-hand woven non-absorbable sutures 5/0; Ethicon, Johnson & Johnson, NY, USA). The pumps released 2.4 IU anti-Xa/h LMWH for at least 14 days. Mice were sacrificed at 21 days after cancer cell inoculation and livers were harvested.

**Measurement of anti-Xa plasma levels**

LMWH plasma levels cannot be determined directly. To have an indication of LMWH plasma levels after treatment, we applied an indirect method by measuring inhibition of factor Xa (anti-Xa) activity [25]. Mouse blood samples were collected in citrate-containing syringes, centrifuged (15 min, 250 g, 4°C) and plasma was stored at –80°C until analysis. Anti-Xa activity was measured using the Coamatic heparin kit (Chromogenix, Milano, Italy) according to the instructions of the manufacturer. For calibration, LMWH in normal mouse plasma was used.

**Tissue preparation**

After the animals were sacrificed at day 21, livers were removed immediately and the surfaces of the liver lobes were macroscopically examined for the presence of tumors. The size of all tumor nodules present was measured. The livers were weighed and subsequently frozen in aluminium boxes in liquid nitrogen and stored at –80°C. Serial cryostat sections (6 μm thick) were cut and stored at -20°C until further use.

**Staining procedures**

In vitro experiments have shown that LMWH has distinct effects on fibrin matrix formation
resulting in reduced angiogenesis by human microvascular endothelial cells [26]. Therefore, we have analyzed in particular the composition of the extracellular matrix and the amount of blood vessels in tumors.

Since fibrin is the end product of the coagulation cascade and since LMWH has anticoagulant properties, we hypothesized that there would be less fibrin deposition in tumors of LMWH-treated mice as compared with controls. In order to test this hypothesis, we performed staining of liver sections with an antibody against murine fibrinogen that cross-reacts with fibrin. Since it is possible that both fibrin and fibrinogen was deposited postmortem within the intratumoral vessels due to stasis of the blood in these vessels after death, staining in serial sections was compared with that of blood vessels as detected with endoglin staining and alkaline phosphatase activity staining.

To determine whether vascularization of the tumors was affected by treatment, we stained sections of livers of treated and untreated mice with an antibody against CD105 (endoglin). Endoglin is involved in blood vessel development and represents a powerful marker of neovascularization [27]. It has been shown to be expressed by peritumoral and intratumoral blood vessels in a variety of human cancer types, and to be only rarely expressed by neoplastic cells [28]. Alkaline phosphatase activity can also be used to detect endothelial cells in normal tissue and in some types of tumors [29].

**Histology**

Cryostat sections were air-dried for 10 min at room temp and stained for general morphological analysis using Giemsa or for specific analysis of the extracellular matrix using Shoobridge staining [30,31]. After rinsing in distilled water, sections were air-dried overnight and mounted in Euparal (Chroma, Stuttgart, Germany).

**Immunohistochemical staining**

Cryostat sections were air-dried for 10 min at room temp. Sections were fixed in 4% (v/v) formaldehyde in distilled water for 20 min and rinsed in PBS 3 times afterwards. Successively, endogenous peroxidase was inhibited by incubating sections in 0.3% (v/v) H2O2 and 0.1% (w/v) NaN3 in distilled water for 20 min at room temp. After rinsing in PBS 3 times, sections were incubated in PBS supplemented with 10% (v/v) normal rabbit serum (Dako, Glostrup, Denmark) for 15 min at room temp as a blocking step. This incubation step and all following incubation steps with antibodies were performed in a humidified chamber. A goat monoclonal antibody against mouse fibrinogen that cross-reacts with fibrin (Accurate, Westbury NY, USA) and a rat monoclonal antibody against mouse CD105 (clone M, 7/18; BD Biosciences, Ezembodegem, Belgium) were used as primary antibodies. The antibodies were diluted 1:500 and 1:10, respectively, in PBS in the presence of 1% bovine serum albumin. Sections were incubated for 1 h at room temp. After rinsing in PBS 3 times, the second antibody, for fibrinogen, rabbit anti-goat immunoglobulin coupled to horseradish peroxidase (Dako) diluted 1:100 in PBS and for CD105, rabbit anti rat immunoglobulin coupled to horseradish peroxidase (Dako) diluted...
Chapter 5

1:200 in PBS and in combination with 0.2 % (w/v) bovine serum albumine and 5% (v/v) normal mouse serum (Dako), was applied and sections were incubated for 1 h at room temp. Binding of secondary antibodies was visualized using a solution of 10 mM diaminobenzidin (Fluka, Buchs, Switzerland), 10 mM NaN3, and 4% (v/v) H2O2 in 50 mM Tris-HCl buffer, pH 7.6, for 10 min at room temp. Control incubations were performed in the absence of the primary antibody in the incubation medium. Subsequently, sections were rinsed 3 times in distilled water and counterstained with haematoxylin (‘Z’ stain; Cellpath, Newton Powys, UK) for 5 sec. After 10 min of rinsing in tap water, sections were mounted in glycerin-gelatin.

Alkaline phosphatase activity

Alkaline phosphatase activity was localized as described by Van Noorden and Jonges [32]. Sections of livers of treated and untreated mice were stained for 1h at 37°C. This (extended) incubation period was selected, since pilot experiments had shown that it enabled visualization of smaller vessels as well, without the occurrence of non-specific staining. Control incubations were performed in the absence of substrate (5-bromo-4-chloro-3-indolyl-phosphate). After incubation sections were rinsed with hot tap water (60°C) to rapidly remove all incubation medium, and mounted in glycerin-gelatin.

Statistical analysis

For data that had a normal distribution in 2 groups of mice, a Student’s t-test was performed to compare the 2 groups statistically. When data were not normally distributed, a non-parametric Mann-Whitney test was performed. Values of p<0.05 were considered to indicate significant differences.

Results

Anti-Xa activity in plasma

The therapeutic range of plasma levels of heparins in humans is >0.3 IU anti-Xa/ml for peak levels and >0.1 IU anti-Xa/ml for trough levels. We tried to simulate these levels in our animal model by varying treatment regimens and measurement of anti-Xa plasma levels in mice. In humans, it is sufficient to administer LMWH subcutaneously once daily but this was not the case in mice (data not shown). Mice that received a mini-osmotic pump containing LMWH which released 2.4 IU anti-Xa/h showed a median plasma level of 0.28 IU anti-Xa/ml on day 9 after surgery and pump implantation (range, 0.01-1.0 IU anti-Xa/ml). Control animals and the animals in the treatment group on day 0 had plasma levels of 0.00 IU anti-Xa/ml (range, 0.00-0.07 IU anti-Xa/ml). No bleeding complications were observed throughout the experiments.

Tumor load

Liver metastases of MCA-38 colon cancer cells were not affected in their development by permanent infusion of LMWH via mini-osmotic pumps. Liver weight was 2.0 ± 0.7 g versus
Do anticoagulants affect colon cancer?

Table 1. Data of mice treated with LMWH using an intraperitoneally-implanted micro-osmotic pump and control mice.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>LMWH-treated (n=10)</th>
<th>Controls (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal anti-Xa level IU/mL -median (range)</td>
<td>0.00 (0.00-0.07)</td>
<td>0.00 (0.00-0.04)</td>
<td>0.68</td>
</tr>
<tr>
<td>Anti-Xa level at day 9 IU/mL -median (range)</td>
<td>0.28 (0.01-1.0)</td>
<td>0.03 (0.01-0.16)</td>
<td>0.03</td>
</tr>
<tr>
<td>Liver weight at end of experiment g -mean +/- SD</td>
<td>2.0 +/- 0.7</td>
<td>1.8 +/-0.8</td>
<td>0.60</td>
</tr>
<tr>
<td>Number of metastasis at liver surface no. -mean +/- SD</td>
<td>49 +/-34</td>
<td>48 +/- 43</td>
<td>0.94</td>
</tr>
<tr>
<td>Percentage of tumor of 1 mm or smaller % -mean +/-SD</td>
<td>53 +/- 18</td>
<td>68 +/- 26</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Figure 1. Serial cryostat sections of colon cancer metastases in livers of mice treated with LMWH released by a mini-osmotic pump at a rate of 2.4 IU anti-Xa/h. a-c, Tumor poor in vessels; d-f, tumor rich in vessels as demonstrated by staining of endothelial alkaline phosphatase activity (a, d), immunohistochemical detection of the endothelial marker endoglin (b, e), and immunohistochemical localization of fibrinogen/fibrin (c, f). Bars = 100 μm. (For color figure see page 193)
1.8 ± 0.8 g in LMWH-treated mice and control mice, respectively. Numbers of tumors on the surface of the livers were 49 ± 34 and 48 ± 43 for each group respectively. The percentage of small tumors was not significantly different between the groups (53 ± 18% versus 68 ± 26%, respectively) (Table 1).

**Morphology and vessel density in tumors**

Staining for endoglin and alkaline phosphatase activity both showed endothelial lining in tumors and liver parenchyma (Figure 1). Tumors and regions within tumors varied greatly in vascular density, even within one animal. This was not very different in LMWH-treated mice and control mice.

Fibrinogen/fibrin staining was found in the cytoplasm of hepatocytes and cancer cells in all mice, irrespective treatment (Figure 1). Extracellularly, fibrin/fibrinogen was found partly colocalized with endoglin and alkaline phosphatase activity (Figure 1 d-f) but also in stroma that did not contain vessels (Figure 1 a-c). These localization patterns were not different in LMWH-treated animals and control animals.

**Discussion**

LMWH has significant life-prolonging effects in cancer patients [4, 6]. Double-blind placebo-controlled randomized studies have shown this effect [7-9, 33]. These effects of LMWH in cancer patients may have a great impact in oncology. Not many, if any, FDA-approved drugs have shown such effects in cancer patients, whereas side effects are limited [9]. Therefore, it is important to optimize treatment of cancer patients with LMWH and/or possibly other anticoagulants and maximize their effects. Animal models are needed to mimic the clinically-found effects of LMWH in cancer patients for the elucidation of molecular mechanisms. Effects were studied in clinical trials that included a wide variety of tumor types [7-9]. Because previous studies showed that hypercoaguability due to FVL enhanced melanoma metastasis in mouse lung but had no effect on colon cancer metastasis in mouse liver [22], we hypothesized that colon cancer growth/metastasis is not dependent on the activation status of the blood coagulation cascade. Therefore, the aim of the present study was to investigate whether development of colon cancer metastasis is susceptible to treatment with anticoagulants.

This study showed that MCA-38 colon cancer cells -metastasizing to the liver after inoculation in the portal vein- were not affected by anticoagulants. There was no difference in liver weight, number, and size of metastases between the treated mice and the controls, indicating that anticoagulants do not have an antimitastatic effect on colon cancer in this animal model.

These data are in agreement with data described by Smorenburg et al using a CC531 colon carcinoma rat model [34]. The number, size or morphology of tumors of rats treated with LMWH (once or twice daily) did not differ significantly from the control group. The LMWH administration scheme may explain the lack of effect of LMWH in the latter study, because
LMWH treatment was started after cancer cell inoculation [34]. In the B16 melanoma lung metastasis model it became clear that anticoagulants have to be administered at the time of cancer cell inoculation [10]. Our present study is based on a similar start of the treatment indicating that the lack of effect of LMWH on colon cancer metastasis is caused by other mechanisms.

In agreement with the findings of the present study, Nagawa et al did not find significantly reduced numbers of metastases in a different experimental colon carcinoma model, by continuous administration of unfractionated heparin (UFH) started before cancer cell inoculation [35]. Together with the results of Klerk et al, in which a hypercoagulability state in FVL mice did not affect metastasis of colon cancer in the liver, our present data indicate that colon cancer metastasis is not dependent on the activation of the blood coagulation cascade and therefore is likely not susceptible for the anticancer effects of anticoagulants.

**Acknowledgements**

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Chapter 5

References

Do anticoagulants affect colon cancer?


