The role of blood coagulation in cancer, inflammation and embryonic development
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Citation for published version (APA):
Bruggemann, L. W. (2008). The role of blood coagulation in cancer, inflammation and embryonic development

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In vivo analysis of the antimetastatic effects of anticoagulants on murine cancer cell lines B16, K1735 and CT26

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(submitted)
Abstract

*Background:* Cancer metastasis is facilitated by blood coagulation. Anticoagulants, such as low molecular weight heparins (LMWHs) and hirudin, a specific anti-thrombin inhibitor, reduce metastasis mainly by inhibition of thrombin formation and L- and P-selectin-mediated cell-cell adhesion. However, exact molecular backgrounds are not understood especially whether the effects are dependent on the cancer cell type and the type of anticoagulant.

*Objectives:* The effect of LMWH on the development of K1735 melanoma metastasis in mouse lungs was investigated. We also compared the antimetastatic effects of hirudin on K1735 and B16 melanoma metastasis and CT26 colon cancer metastasis in mouse lung.

*Methods/experimental design:* Metastatic tumors were determined noninvasively each week up to day 21 in all experiments using bioluminescence imaging. Effects of LMWH and hirudin on metastasis of the three cell lines were correlated with the fibrin/fibrinogen content in the tumors, expression of tissue factor (TF), protease activated receptor (PAR)-1 and -4 and CD24, a ligand of L- and P-selectins.

*Results:* LMWH and hirudin did not have effect on K1735 melanoma metastasis in the lungs. Hirudin inhibited metastasis of B16 cells completely but did not affect metastasis of CT26 cells. TF and PAR-4 expression was similar in all three cell lines. PAR-1 and CD24 were hardly expressed by K1735, whereas CT26 cells expressed low levels and B16 high levels of PAR-1 and CD24. Fibrin content of the tumors was not affected by LMWH.

*Conclusion:* The antimetastatic effects of anticoagulants are dependent on the cancer cell type rather than the anticoagulant type. CD24 expression on cancer cells seems required and possibly PAR-1 expression. The molecular mechanisms are important to understand the life-prolonging effects of anticoagulants in cancer patients.
Introduction

One of the most frequent haematological complications in cancer patients is a disordered coagulation. Indeed, the association between cancer and venous thromboembolic events is well recognized. Moreover, cancer cells can produce activators of the coagulation cascade. Therefore, potential beneficial effects of anticoagulants are investigated for prevention or treatment of cancer.

Clinical trials evaluated the effects of low molecular weight heparins (LMWHs) on survival of cancer patients without thrombosis [1-5]. Overall, there was no difference in survival, but three studies [1, 3, 4] defined a priori a subgroup consisting of patients with a relatively good prognosis at enrolment. Patients in this defined subgroup had a significant survival benefit when treated with LMWHs. These clinical reports are supported by experimental in vitro and in vivo studies showing that spontaneous and induced metastasis is significantly inhibited by anticoagulants [6, 7].

However, mechanisms of the effects of anticoagulants on cancer progression and metastasis are still not exactly known. Animal studies showed that different components of the coagulation system, such as tissue factor (TF) [8], factor Xa [9], thrombin [10] and fibrinogen [11] can contribute to tumor progression by promoting metastasis, whereas the TF pathway inhibitor (TFPI) inhibits metastatic tumor growth in mice [12]. Congenital coagulation disorders (FV Leiden mutation, FVL) can promote hematogeneous tumor cell metastasis as well, in contrast to FVIII-deficient (haemophilic) mice who are protected against cancer cell metastasis, indicating a role for the coagulation process [13], but a lack of effects of FVL has been observed as well [14].

The antimetastatic properties of anticoagulants appears to be mediated by various mechanisms [7]. In the present study, we focussed on inhibition of thrombin formation and platelet-cancer cell complex formation by anticoagulants. These processes are closely related. Cancer cells often express TF on their surface which can trigger thrombin formation in combination with the procoagulation activity of platelets. Thrombin is the most potent activator of platelets via proteinase activated receptors (PARs) [15,16]. PAR signalling induces upregulation of adhesion molecules on endothelial cells and thus binding of platelets via P-selectin. Cancer cells can also upregulate P-selectin expression on platelets and endothelial cells, whereas cancer cells can express P-selectin ligands, such as CD24 and Sialyl Lewis x/a- on their plasma membrane [17]. Their interactions result in the formation of platelet-cancer cell complexes that favour survival of cancer cells in the circulation and promote metastasis [18-21] by protecting cancer cells against the immune system and mechanical stress [22]. Anticoagulants, including LMWH and hirudin, prevent binding of platelets to cancer cells [21,23]. Furthermore, thrombin converts fibrinogen into fibrin. Fibrin depositions have been found in and around various types of tumors, providing scaffolding for angiogenesis and possibly protecting cancer cells against the host defence as well [24].
In the present study, we explore underlying mechanisms of the effects of anticoagulants on cancer metastasis by analysing the effects of LMWH and hirudin on metastasis of three different cancer cell lines and fibrin/fibrinogen content in tumors. We also investigated whether cancer cells are depending on thrombin formation or the formation of platelet-cancer cell complexes for survival, by screening the various cancer cell lines for the capacity to form thrombin and expression of surface proteins involved in adhesion.

**Materials and Methods**

**Animals**

For all experiments, eight weeks old Balb-/c nude mice with a body weight of 20-27 g were used (Netherlands Cancer Institute, Amsterdam, The Netherlands). Animals were maintained under constant environmental conditions with free access to food and water. All animal experiments were performed in agreement with the Animal Ethics Committee of the National Cancer Institute.

**Cancer cell lines**

Three mouse cancer cell lines expressing luciferase were used in experimental models of metastasis in mouse lungs. The K1735 mouse melanoma cell line (kindly provided by Dr. I.J. Fidler, MD Anderson Cancer Institute, Houston, TX), the B16 mouse melanoma cell line (American Type Culture Collection, Manassas, VA [25]) and the CT26 mouse colon carcinoma cell line (also kindly provided by Dr. I.J. Fidler) were used. Preparation of cell cultures, transfection with the luciferase gene and inoculation of the cells in the tail vein of mice have been described previously [13,26,27].

**Lung colonization model**

In the first set of experiments, fifteen mice were administrated $3 \times 10^5$ K1735 cells into the tail vein. Ten mice received 600 anti-Xa (aXa) IU of the LMWH, nadroparin (Sanofi-Synthelabo, Berlin, Germany), intraperitoneally per kg body weight, prior to cancer cell inoculation. The other mice received phosphate-buffered saline (PBS) intraperitoneally and served as controls. Mice were sacrificed at 21 days after cancer cell inoculation and lungs were harvested.

In the second set of experiments, thirty mice received $3 \times 10^5$ K1735 cells, twenty mice received $3 \times 10^5$ B16 cells and twenty mice received $3 \times 10^5$ CT26 cells into the tail vein. Half of the mice in each group received 10 mg/kg PEG-hirudin (Abbott, Knoll, Ludwigshafen, Germany) subcutaneously before cancer cell inoculation. The other half of the mice received PBS subcutaneously before cancer cell inoculation. Mice were sacrificed at 21 days after cancer cell inoculation and lungs were harvested.

**Bioluminescence imaging**

For all experiments, cancer cell load in lungs was measured at day 1, 7, 15 and 21 after
administration of the cancer cells using noninvasive bioluminescence (BLI) as previous described [26,27]. To generate BLI by luciferase, its substrate, luciferin (Xenogen, Alameda, CA) was given intraperitoneally to the mice (150 mg/kg body weight). Luciferase converts luciferin into oxyluciferin and the resulting BLI was imaged with the use of a highly sensitive cooled charge-coupled device camera in a lighttight chamber (IVIS Imaging System 100 series; Xenogen). The acquired images were analyzed with the software program Living ImageR 2.11 (Xenogen). The area of the lungs was selected as region of interest (ROI) for the quantification of BLI.

**TF activity**

TF activity was measured of the three cell lines to test their ability to induce coagulation using a standard procoagulant activity assay (also known as the one-stage clotting assay or recalcification assay) [28]. Cancer cells were suspended in PBS at a concentration of 7.5·10⁴ cells/ml and 75 μl of the cell suspensions was added to 75 μl mouse plasma (Sigma, St. Louis, MO) and incubated at 37°C for 2 min. Then, 100 μl of a 25 mM calcium chloride solution was added and the clotting time was measured using a KC-10 coagulometer (Amelung, Lemgo, Germany).

**Histochemistry**

Lungs were analyzed microscopically in order to determine whether LMWH altered fibrin/fibrinogen depositions in the tumors. Cryostat sections (6 μm thick) were cut at a cabinet temperature of -25°C using a motor-driven cryostat (Bright, Huntingdon, UK).

**Fibrin/fibrinogen staining**

Cryostat sections were air dried for 60 min at room temp. Sections were fixed in 4% (v/v) formaldehyde in distilled water for 20 min and rinsed 3 times in PBS afterwards. Then, 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 3 times in PBS, sections were incubated in PBS supplemented with 10% (v/v) normal rabbit serum (Dako, Glostrup, Denmark) for 15 min at room temp as 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 Chemical and Scientific Corp, Westbury, NY) was used as primary antibody. The antibody was diluted 1:500 in PBS in the presence of 1% bovine serum albumin. Sections were incubated for 60 min at room temp. After rinsing 3 times in PBS, the secondary antibody, rabbit anti-goat immunoglobulin coupled to horseradish peroxidase (Dako) diluted 1:100 in PBS in combination with 0.2% (w/v) bovine serum albumin and 5% (v/v) normal mouse serum (Dako) was applied and sections were incubated for 60 min at room temp. Binding of secondary antibodies was visualized using a solution of 10 mM dianminobenzidine (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, the 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.

**Flow cytometry**

Flow cytometric analysis of the three cancer cell lines was performed to determine the presence of various surface proteins. Cancer cells were incubated in the absence of serum during 24 h before flow cytometric analysis. Cancer cells were centrifuged at 400 g for 5 min. Cells were washed twice with flow-activated cell sorter (FACS)-buffer (PBS supplemented with 0.5% bovine serum albumin, 0.01% NaN₃, and 0.35 mM EDTA) and finally suspended in FACS buffer (4·10⁶ cells/ml). For each analysis, at least 1·10⁵ cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Fc receptors were blocked with purified anti-mouse CD16/32 antibodies (dilution, 1:50; Fcy 111/11 receptor; Pharmingen, BD Biosciences, Breda, The Netherlands). Staining was performed using rat anti-mouse CD24 antibody coupled with phycoerythrin (PE) (dilution, 1:50; Pharmingen, BD Biosciences), goat anti-mouse PAR-4 antibody (dilution, 1:50; M-20 Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-mouse PAR-1 (dilution, 1:50; thrombin R; H-111 Santa Cruz Biotechnology). As secondary antibody anti-rabbit coupled with Alexa fluor 488 (dilution, 1:150; Molecular Probes, Eugene, OR) was used to detect PAR-1 expression and anti-goat coupled with FITC (dilution, 1:200, Dako) was used to detect PAR-4. To correct for nonspecific staining all analyses were also conducted with cells stained with the appropriate isotype control antibodies. Cancer cells were identified by forward and sideangle light scatter gating. Data are presented as the difference between mean fluorescence intensities of specifically-stained and nonspecifically-stained cells.

**Statistical analysis**

Results are presented logarithmically as percentages of positive cells (mean +/- SEM). BLI data were compared using the Mann Whitney U-test (unpaired, non-normally distributed groups). Values were given as median. P values <0.05 were considered to indicate significant differences.

**Results**

**Bioluminescence**

The brightest BLI in the mice was found in the ROI, the lungs. BLI was measured weekly. Figure 1 shows the exponential increase in BLI values in the ROI in time. Some mice showed BLI in the tail vein at the site of cancer cell inoculation (Figure 2) and BLI was also found in the abdominal area, indicating cancer spread to abdominal organs.
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**Figure 1:** Noninvasive bioluminescence imaging of an individual mouse at days 1, 7, 15 and 21 after intravenous administration of B16 melanoma cells. The BLI signal as a measure of cancer cell load in the region of interest (ROI) increased from below the detection limit (ND) at day 1 to $0.23 \times 10^5$, $2.6 \times 10^5$ and $110 \times 10^5$ photons/s at days 7, 15 and 21, respectively. (For color figure see page 194)

**Figure 2:** Bioluminescence as visualized noninvasively in an individual mouse at days 7 and 21 after intravenous administration of luciferase-expressing B16 melanoma cells into the tail vein. The mouse shows bioluminescence in the tail indicating the presence of cells at the site of cancer cell inoculation. The region of interest (ROI) for bioluminescence measurements is given in red, showing a cancer cell load of $3.7 \times 10^5$ and $120 \times 10^5$ photons/s, at days 7 and 21 respectively. (For color figure see page 194)
Chapter 6

The effects of LMWH on K1735 melanoma metastasis

Bioluminescent K1735 melanoma cells induced tumors in lungs of mice irrespective treatment. A bolus of LMWH before cancer cell inoculation did not affect the growth rate of the metastases in the lungs. This is confirmed by the bioluminescence values of all mice at day 21 after cancer cell inoculation (Figure 3).

The effect of hirudin on K1735 and B16 melanoma and CT26 colon carcinoma metastasis

Hirudin did not have an effect on the formation of tumors in the lungs by K1735 cells. Mice that were given B16 melanoma cells showed a strong inhibition of the exponential increase in cancer cell load in the lungs in the hirudin group as compared to the control group (Table 1). CT26 colon carcinoma metastasis in the lungs was not affected by hirudin either.

Fibrin/fibrinogen content of tumors

Fibrin/fibrinogen content in K1735 melanoma tumors was similar in LMWH-treated mice and control mice.

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**Figure 3:** Bioluminescence in lungs of mice at day 21 after K1735 melanoma cell inoculation in the tail vein. No significant difference is found between LMWH-treated mice and control mice (bars represent median).

**Table 1:** Bioluminescence in mice at day 21 after B16, K1735 and CT26 cancer cell inoculation. Bioluminescence values are given as photons/s in the region of interest (ROI) (see Figures 1 and 2) (median, interquartile range)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Hirudin</th>
<th>No Hirudin</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16</td>
<td>0 *</td>
<td>200·10⁵ (0.73·10⁵-270·10⁵)</td>
<td>0.04</td>
</tr>
<tr>
<td>K1735</td>
<td>700·10⁵ (73·10⁵-1500·10⁵)</td>
<td>760·10⁵ (190·10⁵-930·10⁵)</td>
<td>0.98</td>
</tr>
<tr>
<td>CT26</td>
<td>1.4·10⁶ (1.2·10⁶-1.8·10⁶)</td>
<td>1.3·10⁶ (0.76·10⁵-2.5·10⁶)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* median
In vivo analysis of the antimitastatic effects of anticoagulants on murine cancer cell lines

**Figure 4:** Tissue factor (TF) activity in cultured cancer cells. TF activity was determined in $7.5 \times 10^4$ cells and expressed as procoagulant activity (PCA) in sec (mean +/- SEM). PCA is inversely related to TF activity.

**Figure 5:** FACS analysis of immunofluorescent staining of PAR-1, PAR-4 and CD24 expression. Fluorescence is shown on the X-axis on a logarithmic scale and percentage positive cells on the Y-axis. PAR-1 was found in significant amounts on B16 and CT26 cells but not K1735 cells, PAR-4 expression was not found in any of the three cell lines. CD24 was expressed on a large part of the B16 cells, a small fraction of CT26 cells and not on K1735 cells. Grey areas represent control cells; black areas represent positive cells.
Chapter 6

TF activity

Procoagulant TF activity of the three cancer cell lines was not significantly different (Figure 4).

CD24, PAR-1 and PAR-4 expression

K1735 cells did not express CD24, PAR-1 and PAR-4, whereas B16 cells expressed both CD24 and PAR-1 but not PAR-4 (Figure 5). CT26 cells expressed PAR-1 and to a limited extent CD24, but not PAR-4.

Discussion

Double-blind placebo-controlled randomized studies have shown a significant life-prolonging effect in cancer patients treated with LMWH [1-3, 5]. Therefore, it is important to optimize treatment of cancer patients with LMWH and/or possibly other anticoagulants and maximize these effects. Murine models of experimental metastasis have been used frequently to investigate the effect of anti-haemostatic agents on blood borne metastasis. Although such artificial models do not encompass the entire metastatic process, they remain useful for ‘proof-of-concept’-experiments, focusing on the haematogenous phase of tumors dissemination. Several studies have demonstrated significant efficacy of anticoagulants [6, 7].

In this study, we determined the effects of an LMWH, nadroparin, on the development of K1735 melanoma metastasis in mouse lungs and of hirudin on the development of K1735, B16 melanoma and CT26 colon cancer metastasis in lungs. In contrast to previous studies, we did not observe any inhibitory effect of LMWH or hirudin on metastasis of K1735 melanoma cells and fibrin/fibrinogen content in tumors. Hirudin - a strong inhibitor of thrombin - was not able to inhibit metastasis in this model and in the CT26 colon cancer metastasis model, whereas the inhibitory effect on B16 metastasis was potent as has been demonstrated before [10]. The different antimetastatic effects of hirudin cannot be explained by differences in thrombin or fibrin production because the TF activity was comparable in the three cancer cell lines. These findings indicate that different mechanisms of metastasis play a role, rather than of the coagulation cascade.

Thrombin binds PAR-1 and mediates increased invasiveness and metastatic potential of cancer cells [29-31] and enhanced cancer cell adhesion to platelets [32-34], endothelial cells [35], fibronectin and Von Willibrand factor [10, 33]. Inhibition of thrombin by hirudin may turn off the PAR-1 signalling resulting in decreased metastasis. Indeed, metastasis of B16 melanoma cells was affected by hirudin and these cells expressed PAR-1 on their surfaces whereas K1735 melanoma cells did not. On other hand, the CT26 cell line also expressed PAR-1 whereas hirudin did not affect metastasis of this cell line. Possibly, both K1735 and CT26 cell lines may not be dependent on the coagulation cascade to metastasize and therefore, are not affected by hirudin.

We also investigated the relevance of PAR-4 expression for metastasis of cancer cells. The role of PAR-4 in cancer is not known, but its expression was upregulated in prostate cancer [36]
suggesting that PAR-4 is involved as well. However, none of the three cell lines that we tested expressed PAR-4, indicating that the antimetastatic effects of hirudin are not depending on this receptor either.

Finally, the difference in the effects of hirudin on metastasis of the cancer cell lines may be related with adherence to platelets via P- and L-selectin, to protect the cancer cells against shear stress and the immune system. Importantly, both platelet-cancer cell aggregation and experimental metastasis of human cancer cells were significantly inhibited in P-selectin-deficient mice [37, 38]. Previous reports indicate that CD24 takes part in metastasis as a ligand for P-selectin. Expression of CD24 as ligand for platelet binding is upregulated by thrombin, indicating that direct inhibition of thrombin may prevent binding of platelets to cancer cells, rendering the cancer cells vulnerable in the circulation [21, 39, 40]. CD24 expression was found on B16 cells, in low levels on CT26 cells and it was absent on K1735 melanoma cells. So, the antimetastatic effects of hirudin on B16 cells can also be explained by inhibition of CD24. A small fraction of CT26 cells expressed CD24 as well but the numbers may have been too small for successful metastasis and thus to show effects of hirudin. CT26 as well as K1735 cells may have been arrested mechanically in the capillaries due to size restriction as was reported by Mook et al for colon cancer metastasis in rats [41] and such a process cannot be inhibited by hirudin.

In summary, the results of our experiments indicate that anticoagulants have their antimetastatic effects on specific cancer cell types. In our model, anticoagulants affect B16 melanoma but not K1735 melanoma and CT26 colon carcinoma metastasis in lungs. K1735 melanoma and CT26 colon carcinoma cell lines must metastasize via other mechanisms, independent of thrombin activation, making them insensitive for hirudin treatment. The B16 melanoma cell line requires a hirudin target for survival, such as selectin-mediated adhesion and may be the most appropriate murine tumor model for research on the effect of coagulation on cancer.

Acknowledgements

We would like to thank Ms. D. Lopes Barreto from the Department of Clinical Chemistry of the Netherlands Cancer Institute for her excellent technical assistance. We also would like to thank Mr. R.D. Liu from the Center for Experimental and Molecular Medicine of the Academic Medical Center for his excellent technical assistance.
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