



Inhibition of pulmonary metastasis in a human MT3 breast cancer xenograft model by dual liposomes preventing intravasal fibrin clot formation

Jane Wenzel, Reiner Zeisig, Wolfram Haider, Sylvia Habedank, Iduna Fichtner

► To cite this version:

Jane Wenzel, Reiner Zeisig, Wolfram Haider, Sylvia Habedank, Iduna Fichtner. Inhibition of pulmonary metastasis in a human MT3 breast cancer xenograft model by dual liposomes preventing intravasal fibrin clot formation. Breast Cancer Research and Treatment, 2009, 121 (1), pp.13-22. <10.1007/s10549-009-0448-4>. <hal-00485059>

HAL Id: hal-00485059

<https://hal.science/hal-00485059v1>

Submitted on 20 May 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Inhibition of pulmonary metastasis in a human MT3 breast cancer xenograft model by dual liposomes preventing intravasal fibrin clot formation

Authors: Jane Wenzel^{±,a,c}, Reiner Zeisig^b, Wolfram Haider^d, Sylvia Habedank^e and Iduna Fichtner^a

Affiliation: a) Max-Delbrück-Center for Molecular Medicine Berlin-Buch;
R.-Rössle-Strasse 10; 13122 Berlin, Germany
b) Experimental Pharmacology and Oncology Berlin-Buch GmbH
(EPO GmbH); R.-Rössle-Strasse 10; 13122 Berlin, Germany
c) Charité', Campus Benjamin Franklin, Medical Clinic IV, Nephrology, Berlin
d) Institute for Animal Pathology; Schönhauser Straße 62, 13127 Berlin
e) Labor Sylvia Habedank, Postfach 49 01 52, 12281 Berlin

Corresponding author: Jane Wenzel[±]
Max-Delbrück-Center for Molecular Medicine Berlin-Buch
R.-Rössle-Strasse 10; 13122 Berlin, Germany
Phone: +49-30-9406 3585
Fax: +49-30-9406 3823
e-mail: j.wenzel@mdc-berlin.de

Running title: Inhibition of intravasal metastases by dual liposomes

Key words: Intravasal micro-metastases; breast cancer; fibrin clot; platelet aggregation; Liposomes; lung metastases

Abstract

The process of metastasis formation in cancer is not completely understood and is the main reason cancer therapies fail.

Previously, we showed that dual liposomes simultaneously containing the haemostatic inhibitor, dipyridamole and the anticancer drug, perifosine potently inhibited metastasis, causing a 90% reduction in the number of lung metastases in a murine experimental metastasis model. To gain deeper insight into the mechanisms leading to the inhibition of metastasis by these dual liposomes, in the present study, the development of metastases by MT3 breast cancer cells in a mouse xenograft model was analyzed in more detail with regard to tumor cell settlement and metastatic growth.

We found that the development of lung metastases by MT3 tumor cells is essentially dependent on the formation of fibrin clots as a precondition for the pulmonary arrest of tumor cells and the subsequent intravascular expansion of micro-metastases before their invasion into the surrounding tissue.

Introduction

About 3.2 million new patients are diagnosed with cancer in the European Union every year, 30% of whom already have detectable metastases. Although cancer metastasis is still the major cause of cancer therapy failure and the reason for the high mortality rate in cancer patients, the underlying processes leading to the formation of metastases are not completely understood.

Metastasis is a complex multi-step process. It involves the detachment of tumor cells from the primary tumor, their penetration into blood vessels, as well as their entry into the circulation and adhesion to the endothelium, allowing tumor cell extravasation into the surrounding tissue and colonization at secondary sites [1-4]. During circulation in the blood stream and resettlement at distant sites, extensive interactions between tumor cells and endothelial cells and/or blood components like platelets occur [5-8]. These interactions can be mediated by specific ligands, like Sialyl-Lewis^X or ^A that are expressed on the surface of tumor cells and P- and E-selectins found on platelets and endothelial cells, respectively [7,9,10].

The important role of platelets in the process of metastasis is well known since Trousseau first described the close correlation between cancer and thromboembolic diseases [11]. Several investigations have demonstrated the significant role platelets play in metastasis by forming aggregates with circulating tumor cells, promoting tumor cell adhesion, and enhancing cancer development and progression [3,7-9]. Tumor cell-induced platelet aggregation results in the development of tumor cell-platelet microemboli [3,5,7,12,13]. These microemboli protect tumor cells from attack by natural killer cells [3,7,13], leading to an enhanced arrest of tumor cell-platelet aggregates in small blood capillaries and facilitating tumor cell invasion into the surrounding tissue [3,5].

Subsequently, single tumor cells migrate into the extracellular tissue [3,14], or tumor growth begins inside the blood vessel until micro-metastases invade the surrounding tissue [15].

Two general therapeutic approaches exist to attack tumor cells migrating in the blood stream; the first is to prevent tumor cells from interacting with the endothelial membrane [16-18], and the second is to inhibit formation of aggregates between platelets and tumor cells [6,9,19,20]. Efforts to prevent tumor cells from adhering to endothelial cells has mostly focussed on a competitive blockade of the E-selectin receptor on vascular endothelial cells [21,22]. In recent studies, we used liposomes bearing Sialyl-Lewis^X-ligands; these prevented the binding of different tumor cell lines to endothelial cells *in vitro*, but failed *in vivo* [23]. A detailed investigation revealed that the presence of ligand-modified liposomes facilitates the assembly of tumor cell-platelet aggregates, which might have enhanced the formation of lung metastases [24].

Borsig et al. showed that aggregate formation between platelets and tumor cells in L- and P-selectin-deficient mice, and the subsequent number of lung metastases in the mice was significantly reduced [7,25]. They also showed that the formation of complexes between tumor cells and platelets is primarily modulated by P-selectin, and that deficiency in P-selectin expression significantly reduced metastasis [7,25]. Thus, preventing or inhibiting aggregate development can be regarded as a new approach to inhibit metastasis [26].

We developed dual liposomes (D/O-L), which simultaneously contain the cancerostatic drug perifosine (OPP) and the hemostatic inhibitor dipyridamole (DIP). These D/O-L completely abolished aggregate formation between tumor cells and platelets *in vitro* and significantly reduced the formation of lung metastases in mice by 90% in a previous study [26].

Despite the convincing therapeutic effect observed in this experimental model of metastasis, how the tumor cells settled and the metastases grew in the lungs of the mice after an intravenous (i.v.) injection of MT3 breast cancer cells remained unclear. Additionally, we wanted to characterize the mechanism of action responsible for the dual liposomes anti-metastatic effect. Our results show that metastasis in the MT3 breast cancer model involves intravasal fibrin clot formation within the capillary system of mouse lungs. This is apparently an initial and essential step for the development of metastases, because preventing fibrin clot formation with a single i.v. injection of D/O-L significantly reduced the number of pulmonary metastases.

Material and Methods

Material

Cell culture material was obtained from Life Technologies (Karlsruhe, Germany) or Biochrom (Berlin, Germany). Collagen, cuvettes, magnetic stirrer and NaCl for aggregometer measurements were purchased from Chrono-Log. Dicetylphosphate and Cholesterol were obtained from Serva (Heidelberg, Germany), DIP from Sigma-Aldrich (Steinheim, Germany). N-Methyl-(polyethyleneglycol)₂₀₀₀-1,2-distearyl-s,n-glycero-3-phosphatidylethanolamine was purchased from Lipomed (Arlesheim, Switzerland). OPP was a generous gift from Dr. Hilgard (ASTA Medica, Frankfurt) and phosphatidylcholine (PC) from Lipoid (Ludwigshafen). Solvents and high performance thin layer chromatography (HPTLC) plates were purchased from Merck (Darmstadt, Germany).

Methods

Preparation and characterization of vesicles

D/O-L and Control-L were prepared as described previously [26]. Briefly, vesicles were prepared by lipid film/hydration method in combination with extrusion through polycarbonate filters of 400 nm pore size. Non-encapsulated DIP was removed by size exclusion chromatography using Sephadex-G50. Vesicle size was determined by photon correlation spectroscopy using a Coulter Counter N5 (Coulter Electronics Inc., Hialeah, USA). Final concentrations of liposomal PC and OPP were determined by HPTLC. DIP was determined by fluorescence measurement at 415 nm (excitation) and 500 nm (emission) wave length as described previously [26].

Cell culture

Human MT3 breast cancer cells [27] were cultured to sub confluence in RPMI medium supplemented with 10% fetal calf serum and 2 mol/ml L-glutamine, at 37°C, 90% humidity

and 5% CO₂. The cells were detached by a brief exposure to trypsin solution (0.25% trypsin, 0.53 mmol EDTA), spun down at 200 g⁻¹ for four minutes, resuspended in medium and counted in a Neubauer chamber for a dilution as indicated.

In vitro experiments

Platelet aggregation: Platelet aggregation was determined with an impedance aggregometer (Chrono-Log) as described previously [26]. For that purpose, human platelets in plasma were mixed with 900 nmol total lipid of D/O-L or Control-L, activated with 5 ng/ml collagen and the aggregation was measured at 37°C. The higher the platelets are activated, the more platelets aggregate in layers around the surface of the electrodes. This platelet layers result in an increase in the electrical resistance between the two electrodes [28]. The maximal aggregation value (amplitude given in ohm) was obtained after 20 min, when a plateau phase was reached.

Complex formation of platelets and tumor cells: Aggregate formation of MT3 tumor cells in the presence of platelets was performed as described previously [26]. Briefly, platelets and fluorescently labeled MT3 cells were mixed with D/O-L or Con-L and thrombin. The formation of tumor cell aggregates was analyzed after 20 min incubation by microscopic quantification as described previously [26]. The results present the mean number of MT3 cell complexes with at least five cells counted in two representative areas with a total of 80-160 tumour cells in each area. Each experiment was performed in duplicate for four different platelet populations.

In vivo mouse experiments

All animal experiments were performed according to the German Animal Protection Law and by approval of the local responsible authorities.

Female NMRI:nu/nu mice and Crl:Balb/c mice were obtained from Taconic Europe, Denmark. The animals were held under pathogen-free conditions in individually ventilated cages under standardized environmental conditions (22°C room temperature, 50 ± 10% relative humidity, 12 hours light-dark rhythm). They received autoclaved food and bedding (Ssniff, Soest, Germany) and acidified (pH 4.0) drinking water ad libitum.

Determination of platelet and leukocyte number and platelet aggregation

Each of four female NMRI:nu/nu-mice received i.v. 5×10^6 human MT3 breast cancer cells in 400 µl PBS or D/O-L in 100 µl PBS (100 nmol total lipid). Control mice only received 400 µl PBS. At the indicated time points between 0.5 hours and 10 days, tail vein blood was taken and immediately mixed with sodium citrate in a volume ratio of 9:1.

Platelet and leukocyte count: The number of platelets and leukocytes in the mouse blood after tumor cell injection was measured with a cell counter device (Coulter Counter).

Platelet aggregation ex vivo: 180 µl tail vein blood of each mouse was mixed with 720 µl 0.9% sodium chloride. Platelet aggregation was determined with an impedance aggregometer (Chrono-Log) as described above. Aggregation was measured for 20 min at 37°C after activation with 5 ng/ml collagen.

Analysis of tumor cell settlement in mouse lungs

Each of four female NMRI:nu/nu-mice per experimental group was i.v. injected with 5×10^6 MT3 breast cancer cells. Two out of these four mice were additionally treated with 100 nmol total lipid of D/O-L six hours before tumor cell injection. Mice were sacrificed at the indicated time points and the lungs were fixed in 5% formalin solution.

Paraffin embedding and hematoxylin/eosin staining of lung sections:

Half of the lungs embedded in paraffin, were cut into slices with a thickness of 2-3 µm using a rotation microtome and every third section was stained with hematoxylin/eosin.

The lung sections were covered with synthetic mounting media (Bio Mount; Bio Optica) and analyzed with a fluorescence microscope BX 51 (Olympus). Pictures were taken at 20 or 40-fold magnification.

Detection of tumor cells and fibrin(ogen) in paraffin lung sections:

For fibrin(ogen) detection, paraffin lung sections were deparaffinized with pepsin-solution (10 min at 37°C) and incubated with a polyclonal rabbit anti-mouse fibrinogen antibody (Oxford Biomedical Research) diluted 1:4,000. Then, the sections were incubated with a biotinylated goat anti-rabbit antibody (Biocare) for 45 min at room temperature (RT), followed by an incubation with peroxidase-labeled streptavidine (Zytochem Plus Kit; Zytomed Systems) for 10 min (RT). The visualization was done with the substrate AEC (3-component-system; Zytomed Systems) after 10 min incubation (RT). Finally, sections were stained in haematoxylin (Richard Allen) and embedded in Aquatex (Merck).

For detection of tumor cells, the paraffin slides were deparaffinized by heat-induced antigen-demasking with citrate buffer (pH 6) for 5 min at 120°C. Human MT3 tumor cells were stained using the monoclonal Pan Cytokeratin antibody mouse anti-human CK AE1/3 (Zymed laboratories) in a 1:400 dilution (45 min incubation).

Spontaneous metastasis in vivo

Female Crl:Balb/c mice received 1×10^4 murine 4T1 breast cancer cells transplanted into the mammary fat pad. Primary tumor growth was observed over the whole experimental period. Mice were treated i.v. with 100 μ l liposome suspension containing 100 nmol total lipid of D/O-L 6 h before tumor cell inoculation and then daily for two weeks. Control mice were treated with PBS. In additional control groups, mice received 100 μ l liposomes containing one or none of the drugs, or the free drugs DIP and OPP together, all in drug concentration corresponding to that in D/O-L. Mice were sacrificed after 30 days and the

number of metastases in mouse lungs were determined. This experiment was performed twice with a total of 16 mice per experimental group.

Statistical evaluation

Statistical comparisons of the data were performed with the unpaired Student's *t*-test (two populations). Differences were considered to be significant at $p < 0.05$.

Results

We first injected mice with a single bolus of human MT3 tumor cells to determine the effect on the number and the physiological properties of mouse platelets. We found that the i.v. injection of MT3 tumor cells reduced the number of platelets by 40% as soon as 30 min after the injection. The decreased platelet count persisted for more than one day. After three days, the platelet count recovered and was comparable to that of controls (Fig. 1). The same effect was observed for leukocytes, with a 50% reduction in the number of white blood cells (Fig. 1).

We prepared dual liposomes (D/O-L) that simultaneously contain OPP and DIP. As a control, liposomes containing no drugs but with the same lipid composition were prepared (Con-L). When human platelets were mixed with 900 nmol of D/O-L or Con-L, and activated with 5 ng/ml collagen at 37°C, the D/O-L significantly reduced the aggregation of human platelets *in vitro* by 60%, whereas empty Con-L had no effect on platelet aggregation (Fig. 2a). These data are consistent with results obtained after i.v. injection of 100 nmol D/O-L into mice. The *ex vivo* aggregation measurement in mouse blood showed that the ability of the mouse platelets to aggregate was reduced by 90% six hours after liposome injection (Fig. 2b).

Next, we determined whether complex formation between MT3 breast cancer cells and activated platelets was affected by liposomes (Fig. 3). The mean number of MT3 tumor cell complexes (consisting of five or more tumor cells) was significantly increased from 2.3 ± 2.0 to 5.9 ± 3.2 aggregates in the presence of thrombin-activated platelets. Complex formation was completely abolished by D/O-L. In the presence of D/O-L, the mean number of MT3 tumor cell aggregates was 1.8 ± 1.3 , which is comparable to the mean number of complexes observed in the presence of non-activated platelets. The Con-L had no effect on the number of the complexes formed (Fig. 3). Additionally, the size of the tumor cell

aggregates, defined as the number of complex-bound tumor cells in each microscopic field was determined. The number of complex-bound tumor cells was significantly elevated from 12.8 ± 10.6 for non-activated platelets to 52.9 ± 22.7 after thrombin activation. In the presence of non-activated platelets, a minimum of 0 and maximum of 33 aggregate-bound tumor cells per microscopic field were counted. In contrast, a minimum of 27 and a maximum of 103 aggregate-bound tumor cells per microscopic field were detected in the presence of thrombin-activated platelets. In the presence of D/O-L, the mean number of aggregate-bound tumor cells was only 12.1 ± 9.5 per microscopic graph, with minimum and maximum values of bound tumor cells between 0 and 32, respectively.

The ability of murine platelets to aggregate was determined after i.v. injection of MT3 tumor cells into mice. Figure 4a shows that collagen-activated platelets from tumor cell-free control mice resulted in a mean amplitude of 18 ohm measured with an impedance aggregometer *ex vivo*. Tumor cell inoculation led to a 40% reduction in mouse platelet aggregation if blood was collected three to six hours after tumor cell injection. The lowest aggregation values were observed after six hours. When mice were treated with D/O-L six hours before MT3 tumor cell injection, the ability of platelets to aggregate was reduced by 80% in comparison with tumor cell-injected mice with a mean amplitude of two ohm (Fig. 4b).

Additionally, the recalcification time (blood clotting time) of mouse blood after i.v. inoculation of MT3 breast cancer cells into mice was determined *ex vivo*. The blood clotting time was significantly prolonged by 45% 30 min after tumor cell injection and returned to the level of controls after 24 hours (data not shown).

To characterize the mode of tumor cell settlement and the formation of metastases in the mouse lung in the MT3 breast cancer xenograft model, mice were sacrificed at different

time points after injection of MT3 breast cancer cells into the lateral tail vein, and lung sections were prepared.

Microscopic analysis of the lungs showed that the lungs of control mice exhibited no alteration in hemostatic status and no fibrin clots were observed in the blood vessels (Fig. 5a). In contrast, fibrin clots were found in the lungs of mice injected with tumor cells within 30 min after injection. These clots occluded the entire blood vessel and entrapped single tumor cells (Fig. 5b). The presence of fibrin(ogen) and tumor cells was confirmed by immunohistological staining with rabbit anti-mouse fibrinogen antibody (Fig. 3c) and mouse anti-human CK AE1/3 antibody (Fig. 5d), respectively. Fibrin clots that did not contain tumor cells disappeared after six hours.

On day five, intravascular growing micro-metastases were found, with some of the tumor cells in a mitotic state (Fig. 5e). Fibrin-staining of the lung sections confirmed that the micro-metastases were still infiltrated with (Fig. 5e) and surrounded by (Fig. 5f) fibrin. Large micro-metastases filled the inner vessel spaces, destroying the endothelial lining and invading the surrounding tissue (Fig. 5g). In our study, the tumor cells were always trapped in fibrin clots, and single tumor cell endothelialization towards the surrounding tissue was never observed.

In mice treated with one i.v. dose of D/O-L before an i.v. injection of MT3 tumor cells, single tumor cells were found at time points up to 48 hours, but they apparently did not survive. On day five, we only observed one pulmonary intravascular micro-metastases surrounded by a few fibrin polymers in one of six mice treated with liposomes.

Using D/O-L in four independent experiments in the experimental MT3 breast cancer metastasis model, a 90% reduction in the number of pulmonary and a 60% reduction in extrapulmonary metastases was obtained with a single D/O-L treatment six hours before tumor cell inoculation [26].

Additionally, a spontaneous model for breast cancer metastasis was used in this study to prove the anti-metastatic effect of the D/O-L in a clinically more relevant model.

Therefore, murine 4T1 breast cancer cells were transplanted into the mammary fat pad of mice. After two weeks the primary mammary tumors had sizes of around 0.23 cm³ when tumor cells started to metastasize to the lungs (data not shown). We found that the treatment of mice with 100 nmol total lipid of D/O-L 6 h before tumor cell inoculation and then daily for two weeks significantly reduced the mean number of pulmonary metastasis by 65% in comparison to the control and to drug-free Con-L (Fig. 6). The combination of the free drugs dipyridamole and OPP together also reduced the mean number of metastases significantly by 33%, although the liposomal encapsulated drugs (D/O-L) were significantly better than the free drugs. OPP-L, DIP-L and Con-L did not reduce the number of metastases formed in mouse lungs (Fig. 6).

Discussion

The objective of this study was to investigate the mechanism of the anti-metastatic effect of dual liposomes containing the hemostatic inhibitor, dipyridamole and the cytotoxic drug perfosine (OPP), in an experimental model of metastasis. Human MT3 breast cancer cells formed distinct metastatic foci, mostly in the lungs, after a single bolus injection into the mouse tail vein.

The direct inoculation of tumor cells into the circulation does not completely mimic the metastatic process, since it bypasses the first steps in metastasis, e.g. the detachment of single tumor cells from the primary tumor [29,30]. On the other hand, an experimental metastasis model like this is suitable for investigating therapeutic mechanisms of action against circulating tumor cells, because the number of tumor cells in the circulation at a defined time point is standardized [29].

There are several hypotheses regarding how tumor cells interact with blood cells, attach to the endothelium, and migrate to the extra-cellular matrix to form metastases. Several studies described the ligand-based adhesion of single tumor cells to the endothelial membrane as an essential and initial step for tumor cell arrest [5,16,31]. Saiki et al. suggested that the tumor cell may roll along the endothelium before it becomes tightly bound to the surface and extravasates into the adjacent tissue [16]. Other studies led to the proposal that close interactions between circulating tumor cells and platelets are an essential step during metastasis [7,8,12,32]. The accumulation of such tumor cell-platelet microemboli in narrow capillaries can facilitate tumor cell invasion into the surrounding tissue [2,5].

Several studies showed that circulating tumor cells could cause an activation and aggregation of platelets [3,33,34]. Our data also suggest that platelets play a direct role during development of micro-metastases in the MT3 model, because the blood clotting

time was found to be significantly longer and platelet aggregation was significantly impaired *ex vivo* after MT3 breast cancer cell inoculation into nude mice. This might originate, in part, from the fact that platelets have already been activated to some extent by circulating tumor cells. These activated platelets are not able to fully respond to an additional activator like collagen used to activate platelets during aggregometer measurements (Fig. 4). On the other hand, the number of platelets in mouse blood was reduced immediately after injection of the tumor cells, as shown in Figure 1.

A similar effect was found six hours after i.v. treatment of mice with D/O-L. There we found again a 90% reduction in the aggregability of the mouse blood platelets by *ex vivo* aggregometer measurements (Fig. 2b). Several studies showed that an impaired platelet aggregation or a reduced platelet number results in the reduction of metastasis [35]. Our data suggest a direct role of platelets in metastasis in the MT3 model, because the inhibition of platelet aggregation by the dual D/O-L shown here, is accompanied by a significant reduction in the formation of pulmonary metastases seen after D/O-L treatment of mice.

Muschel's group conducted comparable studies on tumor cell-platelet interactions and metastasis formation. These investigators suggested that the initial proliferation of tumor cells proceeds inside blood vessels [15]. They proposed a modified model of the development of pulmonary metastases, which includes the following steps: (1) ligand-mediated attachment of tumor cells to the lung endothelial membrane; (2) apoptosis of the majority of the attached tumor cells; (3) intravascular proliferation of the surviving tumor cells assisted by fibrin clots and platelets; and (4) extravasation of micro-metastatic foci into the surrounding tissue [36]. Additionally, they performed experiments using a spontaneous metastasis model and different cell types [15,31] to confirm these data [36].

Our findings are consistent with those of Muschel's group, except that in our model the settlement of tumor cells in the vasculature does not seem to be mediated through a direct interaction with the endothelial membrane. We found single MT3 cells in the center of fibrin clots, but never directly bound to the endothelial surface.

Moreover, our histological studies showed that massive fibrin clots were formed in pulmonary blood vessels directly after i.v. injection of MT3 breast cancer cells, and that these disappeared after six hours. Only tumor cells entrapped in fibrin clots were stable for several days, probably because they enabled tumor cell survival and proliferation in a protected environment with easy access to oxygen and nutrients without the need for neo-angiogenesis, as well as shielding the tumor cells from shear forces.

In a previous study, we showed that co-incubation of platelets and tumor cells with D/O-L abolished complex formation *in vitro*, and inhibited metastasis *in vivo* [26]. The mechanism whereby the D/O-L reduced metastasis was now explained more in detail in the present study.

We demonstrated here that a single dose of D/O-L completely abolished the formation of pulmonary fibrin clots and subsequently significantly reduced the number of lung metastases. It was shown before that the fibrin clots protect the tumor cells from natural killer cell attack [35]. So, the prevention of fibrin clot formation by the D/O-L might enable an easier access of immune cells to the tumor cells facilitating their elimination from the blood stream [35] that resulted in a reduced metastases formation.

It was also shown by the group of Muschel that murine 4T1 breast cancer cells metastasize as single cells to the mouse lungs in a spontaneous metastasis model [36].

In the current study we used, additionally to the experimental MT3 breast cancer xenograft model, this clinically more relevant 4T1 breast cancer model for spontaneous metastasis, to verify the anti-metastatic effect of the D/O-L.

Therefore, murine 4T1 breast cancer cells were transplanted into the mammary fat pad of mice resulting in a primary mammary tumor that was shown to metastasize via the hematogenous route to the lungs and later to other sites, like liver, bone and brain [37,38]. The 4T1 model metastasizes effectively to sites affected in human breast cancer and therefore displays the metastatic characteristics similar to those observed in cancer patients. We found that a daily treatment of mice with D/O-L for two weeks significantly reduced the mean number of pulmonary metastases by 65% in comparison to the control and to drug-free Con-L (Fig. 6). So, we could confirm the efficiency of the dual liposomes in a clinically more relevant model for metastasis.

This study showed that the formation of fibrin clots and the subsequent development of intravascular micro-metastases are important and essential steps in the formation of pulmonary metastases by MT3 tumor cells in the used experimental metastasis model.

It was demonstrated here that the prevention of fibrin clot formation by the D/O-L entails the inhibition of pulmonary metastases in the MT3 model.

D/O-L containing dipyridamole and perifosine is a new drug formulation that potently inhibits metastasis, suggesting that it may be a promising novel drug for future developments.

Acknowledgements

This study was supported by the Federal Ministry of Education and Research of Germany (Biochance PLUS program; PTJ-BIO/0313601). We thank Prof. M. van der Giet for supporting the PhD work of J.W. at the Charite Berlin; M. Becker and M. Lemm (MDC Berlin-Buch) for excellent performance of the animal experiments; and Lipoid GmbH Ludwigshafen for providing us with egg phosphatidylcholine.

Conflict of interest statement

All authors confirm that they have no potential conflict of interest, including any financial, personal, or other relationships with other people or organizations within that could inappropriately influence (bias) their work.

Legends

Figure 1: Change in platelet and leukocyte number after tumor cell inoculation

5×10^6 MT3 breast cancer cells were injected i.v. into NMRI:nu/nu mice. Tail vein blood was taken at the indicated time points. Shown is the mean number of platelets and leukocytes S.D. for four mice per time point.

(*): significantly different to time point “0 h” ($p < 0.05$).

Figure 2: Effect of D/O-L on platelet aggregation *in vitro* and *ex vivo*

a: Aggregation of human platelets in vitro

Platelet aggregation in the presence of D/O-L or Con-L was determined with an impedance aggregometer as described in Material and Methods. Given is the mean maximal amplitude S.D., determined after 20 min measurement for five independently performed experiments, each done in duplicate.

(*): significantly different to “Control” and “Con-L” ($p < 0.05$).

b: Aggregation of mouse platelets ex vivo after in vivo liposome injection into mice

Mouse blood was taken at the indicated time points after i.v. injection of 100 nmol total lipid of D/O-L and maximal platelet aggregation was measured with an impedance aggregometer as described in Material and Methods. Shown is the mean maximal amplitude S.D. obtained after 20 min measurement for four mice/group.

(*): significantly different to time point “0 h” ($p < 0.05$).

Figure 3: Complex formation of MT3 tumor cells in the presence of platelets and liposomes *in vitro*

Fluorescently labeled MT3 cells were incubated with platelets (Pt) or thrombin-activated platelets (aPt) and 100 nmol total lipid of D/O-L or Con-L for 20 min and the mixture was spun down to glass slides. Aggregate formation of tumor cells was determined by counting tumor cell aggregates (> 5 MT3 cells) in representative microscopic areas. Shown is the mean number of complexes S.D. per evaluated micrograph field, whereas the minimal and maximal number of aggregates are displayed in the figure bars.

(*): significantly different to “Pt” and “aPt+D/O-L” ($p < 0.05$).

Figure 4: Effect of i.v. MT3 tumor cell inoculation on platelet aggregation *ex vivo*

a: 5×10^6 MT3 breast cancer cells were injected into NMRI:nu/nu mice. Tail vein blood was taken at the indicated time points. Shown is the mean maximal amplitude of platelet aggregation S.D., determined *ex vivo* after 20 min measurement with an impedance aggregometer for four mice per time point as described in Material and Methods.

(*): significantly different to control ($p < 0.05$).

b: Mice were treated with 100 nmol D/O-L or 100 μ l PBS six hour before inoculation of 5×10^6 MT3 breast cancer cells. Tail vein blood was taken one hour after tumor cell injection. Shown is the mean maximal amplitude of platelet aggregation S.D. with and without D/O-L treatment for four mice per group. Aggregation was measured with an impedance aggregometer as described in Material and Methods.

(**): significantly different to “without D/O-L” ($p < 0.05$).

Figure 5: Pulmonary intravasal metastases formation after i.v. injection of MT3 breast cancer cells

5×10^6 MT3 breast cancer cells were injected into NMRI:nu/nu mice, which were sacrificed at the indicated time points. The lungs were prepared as described in Material and Methods and analyzed microscopically.

Shown are the following lung sections (in brackets: staining of lung section and magnification): **(a)** Control lung; arrow: blood vessel with separate erythrocytes (HE; 20); **(b)** Lung section 30 min after tumor cell inoculation; arrow: single tumor cell in fibrin occluded blood vessel (HE; 20); **(c)** Lung section 30 min after tumor cell injection; arrow: a single tumor cell is trapped inside a fibrin clot (rabbit anti-mouse fibrinogen antibody; 20); **(d)** Lung section 30 min after tumor cell inoculation; arrows: two single tumor cells are located inside a small blood capillary (mouse anti-human CK AE1/3 antibody; 40); **(e)** Five days after tumor cell injection; a micro-metastases is surrounded and infiltrated by fibrin; arrow: mitotic cell (HE; 40); **(f)** Five days after tumor cell injection; arrow: a micro-metastases has developed, which is surrounded by fibrin (rabbit anti-mouse fibrinogen antibody; 20); **(g)** Five days after tumor cell administration; a micro-metastases (blue arrows) has outgrown the blood vessel (black arrow) and invaded the surrounding tissue (HE; 20).

Figure 6: Effect of D/O-L on spontaneous metastases formation in the 4T1 breast cancer model

1×10^4 murine 4T1 breast cancer cells transplanted into the mammary fat pad of Crl:Balb/c mice. Mice were treated i.v. with 100 nmol total lipid of D/O-L 6 h before tumor cell inoculation and then daily for two weeks. Control mice were treated with PBS. Additional control groups received liposomes containing one (OPP-L; DIP-L) or none (Con-L) of the drugs, or the free drugs DIP and OPP together (DIP+OPP), all in drug concentration corresponding to that in D/O-L. Mice were sacrificed after 30 days and the number of metastases was determined in the lungs. Given is the mean percentage of formed metastases in relation to the control (100% metastases) S.D.

(*): significantly different to all groups ($p < 0.05$).

(**): significantly different to "Control" and "D/O-L" ($p < 0.05$).

References

1. Fidler IJ (1990) Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res* 50: 6130-6138.
2. Price JT, Thompson EW (2002) Mechanisms of tumour invasion and metastasis: emerging targets for therapy. *Expert Opin Ther Targets* 6: 217-233.
3. Tsuruo T, Fujita N (2008) Platelet aggregation in the formation of tumor metastasis. *Proc Jpn Acad Ser B Phys Biol Sci* 84: 189-198.
4. Khanna C, Hunter K (2005) Modeling metastasis in vivo. *Carcinogenesis* 26: 513-523.
5. Honn KV, Tang DG, Crissman JD (1992) Platelets and cancer metastasis: a causal relationship? *Cancer Metastasis Rev* 11: 325-351.
6. Felding-Habermann B (2003) Targeting tumor cell-platelet interaction in breast cancer metastasis. *Pathophysiol Haemost Thromb* 33 Suppl 1: 56-58.
7. Borsig L (2008) The role of platelet activation in tumor metastasis. *Expert Rev Anticancer Ther* 8: 1247-1255.
8. Chen M, Geng JG (2006) P-selectin mediates adhesion of leukocytes, platelets, and cancer cells in inflammation, thrombosis, and cancer growth and metastasis. *Arch Immunol Ther Exp (Warsz)*.
9. Borsig L, Wong R, Feramisco J et al (2001) Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc Natl Acad Sci U S A* 98: 3352-3357.
10. Kannagi R (1997) Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. *Glycoconj J* 14: 577-584.
11. Trousseau A (1993) Not Available. *Bull Soc Liban Hist Med* 16-20.
12. Tohgo A, Tanaka NG, Ogawa H (1986) Platelet-aggregating activities of metastasizing tumor cells. IV. Effects of cell surface modification on thrombin generation, platelet aggregation and subsequent lung colonization. *Invasion Metastasis* 6: 58-68.
13. Nieswandt B, Hafner M, Echtenacher B et al (1999) Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res* 59: 1295-1300.
14. Gupta GP, Massague J (2004) Platelets and metastasis revisited: a novel fatty link. *J Clin Invest* 114: 1691-1693.

15. Al-Mehdi AB, Tozawa K, Fisher AB et al (2000) Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat Med* 6: 100-102.
16. Saiki I, Koike C, Obata A et al (1996) Functional role of sialyl Lewis X and fibronectin-derived RGDS peptide analogue on tumor-cell arrest in lungs followed by extravasation. *Int J Cancer* 65: 833-839.
17. Kragh M, Loechel F (2005) Non-anti-coagulant heparins: a promising approach for prevention of tumor metastasis (review). *Int J Oncol* 27: 1159-1167.
18. Hejna M, Raderer M, Zielinski CC (1999) Inhibition of metastases by anticoagulants. *J Natl Cancer Inst* 91: 22-36.
19. Bobek V, Kovarik J (2004) Antitumor and antimetastatic effect of warfarin and heparins. *Biomed Pharmacother* 58: 213-219.
20. Mancuso A, Sternberg CN (2006) New treatment approaches in metastatic renal cell carcinoma. *Curr Opin Urol* 16: 337-341.
21. Gosk S, Gottstein C, Bendas G (2005) Targeting of immunoliposomes to endothelial cells expressing VCAM: a future strategy in cancer therapy. *Int J Clin Pharmacol Ther* 43: 581-582.
22. Kessner S, Krause A, Rothe U (2001) Investigation of the cellular uptake of E-Selectin-targeted immunoliposomes by activated human endothelial cells. *Biochim Biophys Acta* 1514: 177-190.
23. Zeisig R, Stahn R, Wenzel K et al (2004) Effect of sialyl Lewis X-glycoliposomes on the inhibition of E-selectin-mediated tumour cell adhesion in vitro. *Biochim Biophys Acta* 1660: 31-40.
24. Keil C, Zeisig R, Fichtner I (2005) Effect of surface modified liposomes on the aggregation of platelets and tumor cells. *Thromb Haemost* 94: 404-411.
25. Borsig L, Wong R, Hynes RO (2002) 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 U S A* 99: 2193-2198.
26. Wenzel J, Zeisig R, Fichtner I (2009) Inhibition of breast cancer metastasis by dual liposomes to disturb complex formation. *Int J Pharm* 370: 121-128.
27. Naundorf H, Rewasowa EC, Fichtner I (1992) Characterization of two human mammary carcinomas, MT-1 and MT-3, suitable for in vivo testing of ether lipids and their derivatives. *Breast Cancer Res Treat* 23: 87-95.
28. Cardinal DC, Flower RJ (1980) The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J Pharmacol Methods* 3: 135-158.
29. Ottewell PD, Coleman RE, Holen I (2006) From genetic abnormality to metastases: murine models of breast cancer and their use in the development of anticancer therapies. *Breast Cancer Res Treat* 96: 101-13.

30. Fantozzi A, Christofori G (2006) Mouse models of breast cancer metastasis. *Breast Cancer Res* 8: 212.
31. Im JH, Fu W, Wang H et al (2004) Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. *Cancer Res* 64: 8613-8619.
32. Gasic GJ (1984) Role of plasma, platelets, and endothelial cells in tumor metastasis. *Cancer Metastasis Rev* 3: 99-114.
33. Beviglia L, Stewart GJ, Niewiarowski S (1995) Effect of four disintegrins on the adhesive and metastatic properties of B16F10 melanoma cells in a murine model. *Oncol Res* 7: 7-20.
34. Oleksowicz L, Mrowiec Z, Schwartz E (1995) Characterization of tumor-induced platelet aggregation: the role of immunorelated GPIb and GPIIb/IIIa expression by MCF-7 breast cancer cells. *Thromb Res* 79: 261-274.
35. Palumbo JS, Talmage KE, Massari JV et al (2005) Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood* 105: 178-185.
36. Wong CW, Song C, Grimes MM et al (2002) Intravascular location of breast cancer cells after spontaneous metastasis to the lung. *Am J Pathol* 161: 749-753.
37. Tao K, Fang M, Alroy J et al (2008) Imagable 4T1 model for the study of late stage breast cancer. *BMC Cancer* 8: 228.
38. DuPre' SA, Hunter KW, Jr. (2007) Murine mammary carcinoma 4T1 induces a leukemoid reaction with splenomegaly: association with tumor-derived growth factors. *Exp Mol Pathol* 82: 12-24.