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ORIGINAL ARTICLE

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Photodynamic drug delivery enhancement in tumours does not depend on leukocyte–endothelial interaction in a human mesothelioma xenograft model†

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Abstract

OBJECTIVES: The pre-treatment of tumour neovessels by low-level photodynamic therapy (PDT) improves the distribution of concomitantly administered systemic chemotherapy. The mechanism by which PDT permeabilizes the tumour vessel wall is only partially known. We have recently shown that leukocyte–endothelial cell interaction is essential for photodynamic drug delivery to normal tissue. The present study investigates whether PDT enhances drug delivery in malignant mesothelioma and whether it involves comparable mechanisms of actions.

METHODS: Human mesothelioma xenografts (H-meso-1) were grown in the dorsal skinfold chambers of 28 nude mice. By intravital microscopy, the rolling and recruitment of leukocytes were assessed in tumour vessels following PDT (Visudyne® 400 μg/kg, fluence rate 200 mW/cm^2 and fluence 60 J/cm^2) using intravital microscopy. Likewise, the distribution of fluorescently labelled macromolecular dextran (FITC-dextran, MW 2000 kDa) was determined after PDT. Study groups included no PDT, PDT, PDT plus a functionally blocking anti-pan-selectin antibody cocktail and PDT plus isotype control antibody.

RESULTS: PDT significantly enhanced the extravascular accumulation of FITC-dextran in mesothelioma xenografts, but not in normal tissue. PDT significantly increased leukocyte–endothelial cell interaction in tumour. While PDT-induced leukocyte recruitment was significantly blunted by the anti-pan-selectin antibodies in the tumour xenograft, this manipulation did not affect the PDT-induced extravasation of FITC-dextran.

CONCLUSIONS: Low-level PDT pre-treatment selectively enhances the uptake of systemically circulating macromolecular drugs in malignant mesothelioma, but not in normal tissue. Leukocyte–endothelial cell interaction is not required for PDT-induced drug delivery to malignant mesothelioma.

Keywords: Photodynamic therapy • Drug delivery • Intravital microscopy • Dorsal skinfold chamber • Leukocyte • Malignant pleural mesothelioma

INTRODUCTION

Treatment of patients suffering from malignant pleural mesothelioma (MPM) remains a challenge for thoracic surgeons. A multimodality approach combining cytoreductive surgery with adjuvant therapies is now feasible with reasonable morbidity and mortality [1]. To date, extrapleural pneumonectomy combined to chemo- and/or radiotherapy represents the only therapeutic procedure that may be associated with long-term survival in a selected group of patients [2]. Nevertheless, MPM still has a very poor prognosis. The natural course of the disease is characterized by locoregional recurrence and relentless progression in the chest cavity, eventually leading to tumour invasion of the chest wall and the mediastinum.

For that reason, innovative treatment strategies are sought to achieve local tumour control, such as intracavitary chemotherapy [3] as well as intraoperative photodynamic therapy (PDT) of the

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chest cavity after extrapleural pneumonectomy [4, 5]. Intracavitary chemotherapy with intraoperative local application of the cytostatic drug solution into the chest cavity aims to enhance local drug delivery and to improve tumour control at the site of resection, but its widespread clinical application has been hampered by nephrotoxicity following systemic absorption of the cytostatic drug [3]. PDT as adjunct to extrapleural pneumonectomy makes use of a photosensitizer which is administered systemically and subsequently activated by irradiation with laser light in the chest cavity [6]. The resulting photochemical reaction generates cytotoxic products, which results in local tumour cell destruction. PDT of the chest cavity in patients with MPM has been shown to result in tumour destruction [6]; however, depending on treatment conditions, important side effects were reported. The major drawback of cytotoxic PDT combined with extrapleural pneumonectomy is the addition of surgical and PDT-related toxicity [4, 7]. The enhanced morbidity of the procedure was mainly related to the lack of sufficient tumour selectivity of the PDT effect, which, at the applied conditions, leads to destruction of tumour and some vital structures in the chest cavity. Unfortunately, modifications of PDT treatment modalities, including improvements of light delivery, dosimetry to the chest cavity, selection of sensitizers or modification of treatment protocols, have not yielded significant clinical benefits [6].

Snyder *et al.* [8, 9] as well as our own group have shown that a direct vascular effect of PDT at relatively low light doses may be exploited to increase the uptake of systemically circulating drugs to tumour and other target tissues. This is a new treatment concept termed 'photodynamic drug delivery'. If low-dose PDT of the chest cavity after extrapleural pneumonectomy could facilitate targeted delivery of a circulating cytostatic drug to residual tumour in the chest cavity, local tumour control could theoretically be improved even at reduced systemic cytotoxic drug levels. Photodynamic drug delivery combines low-dose PDT—lacking tumouricidal effect as well as normal tissue toxicity—with systemic intravenous chemotherapy. The concept is founded on the idea that low-dose PDT increases microvessel permeability, hence promoting controlled release of circulating drugs into tissue. *In vitro*, phototoxic effects on endothelial cells induce the formation of endothelial gaps [10]. *In vivo*, assessing photodynamic drug delivery on non-malignant tissue, PDT additionally stimulates leukocyte–endothelial cell interaction and thereby mediates PDT effects on improved drug delivery [8].

In the present study, we set out to assess the effect of low-dose PDT on the uptake of a systemically circulating macromolecular agent into human mesothelioma xenografts and normal tissue, using the dorsal skinfold chamber model and intravital fluorescence microscopy.

MATERIALS AND METHODS

Dorsal skinfold chamber

A custom-built dorsal skinfold chamber was implanted on the back of nude mice (Charles River, France), as previously described [8]. After a recovery period of 48 h, dorsal skinfold chambers were selected based on their vascularization and their limited inflammation (hyperaemia, distortion of vein segments and presence of oedema). All animals were kept in a sterile environment, and the experiments were conducted in accordance with the European Institutional Guidelines for Animal Care and Use.

Tumour model

A human mesothelioma tumour cell line (H-meso-1, Mason, Worchester, MA, USA) was maintained in culture, as previously described [11]. For donor tumour generation, a 0.1 ml cell suspension containing 5×10^7 tumour cells was injected subcutaneously behind the left scapula of the nude mice (Charles River). Tumours were grown up to a diameter of 8 mm. The animals were then sacrificed, and the tumours were cut into chunks that were used for dorsal skinfold chamber implantation. Tumour chunks were prepared by removing the capsule and the necrotic portions of the donor tumour and by cutting the remaining tumour in $2 \times 2 \times 2$ mm cubes that were placed in a Petri dish and immersed in cold phosphate-buffered saline. The chunks were then placed in the centre of the dorsal skinfold chambers. The vascularization of the implanted tumour chunks was checked daily, and the experiments began when a stable perfused vasculature was visible using transillumination microscopy.

Photodynamic therapy protocol

PDT was performed using Visudyne® (Novartis, Hettlingen, Switzerland) at a concentration of 400 μg/kg body weight (b.w.). A drug light interval of 10 min was used to ensure homogeneous perfusion of the photosensitizer within the tumour vasculature. Light administration was performed using an Hg-arc lamp filtered at 420 ± 20 nm (Carl Zeiss 'cube filter set 05', Exc. BP 395-440, DM FT 460, Em. LP470) through a 20× water immersion lens. A homogeneous light dose of 60 J/cm^2 was applied to a circular surface (diameter of 400 μm). The fluence rate was 200 mW/cm^2.

Intravital microscopy

A Carl Zeiss Axiotech Vario 100 microscope was used for *in vivo* observation of the dorsal skinfold chamber. For that purpose, the mouse was placed in a lateral decubitus position inside a plexiglas tube which was positioned under the microscope. The chamber on the back of the mouse was fixed horizontally, so that its position under the microscope allowed for transillumination from the bottom side and at the same time for epi-illumination for fluorescence microscopy. Animals were not anaesthetized during intravital microscopy. Achroplan Carl Zeiss 2.5×/0.0075 and 4×/0.10 Plan Neofluar objectives were used for a large field of view (3 × 3 mm for the 4× objective). A 20×/0.50 water immersion objective was used for PDT excitation and close observation of the capillaries (field of view 600 × 600 μm). Excitation was performed via a filtered 100W HBO103 light source (OSRAM GmbH, Augsburg, Germany), powered through a variable Carl Zeiss FluoArc device that allowed diminishing the light power and thus prevents excessive PDT excitation during data recording. A Uniblitz shutter VS25 with its controller VMM-D1 (Vincent Associates, Rochester, NY, USA) was used to cut off the light from the HBO lamp. Images and video sequences were recorded with an on-chip-amplified electron

multiplier consisting of a back illuminated thinned Peltier-cooled CCD camera (EM-CCD C9100-12, 400–1000 nm, Hamamatsu Photonics, Solothurn, Switzerland) allowing for an up to 2000× amplified signal gain. This set-up allowed to lower the excitation light and thus prevents both PDT and photobleaching using the analysis light. Images and sequences were recorded through the Hamamatsu camera controller with the Hamamatsu HiPic version 7.0 software, producing 16 bit grey level images with a size of 512 × 512 pixels. A Multiscan Rate Converter MSC-12A-HPK (Stack Ltd, Saitama, Japan) was added between the EM-CCD and the controller providing digital and video signals in parallel. Date and time information were superimposed on the video signal with a VTG-33 Video Timer (FOR-A, Tokyo, Japan). Images were recorded on an SR-S388E video recorder (S-VHS; JVC, Yokohama, Japan).

Study design

Twenty-three animals underwent Visudyne-mediated low-level PDT followed 1 h later by the intravenous administration of FITC-dextran (2000 kDa). Eight of these animals had PDT, but no antibody injection (PDT group). In two groups of five animals each, PDT was combined to the administration of a pan-selectin inhibitor (MABS group; $n = 5$) cocktail or to an isotype control antibody (isotype group; $n = 5$). Five of 23 animals had no xenograft implanted in the dorsal skinfold chamber. In these animals, PDT was performed on normal tissue. The control group comprised five additional xenograft-bearing animals undergoing Visudyne injection, but no laser irradiation. For each group, the extravasation of FITC-dextran (2000 kDa) was assessed over a 45 min period by intravital microscopy. In addition, the rolling and recruitment of leukocytes were assessed before, 1 and 2 h after PDT.

Tumour permeability evaluation

The extravasation of FITC-dextran was determined by fluorescence microscopy, using an Hg-arc lamp, in real time. The daily stability of the Hg-arc lamp was tested using a rubis 8Sp3 disc (diameter 12 mm, thickness 1 mm; Hans Stettler, Lyss, Switzerland), as described previously [12]. FITC-dextran (25 mg/ml and 100 mg/kg b.w.) was injected in the tail vein 1 h after PDT. FITC-dextran fluorescence was recorded in regions of interest (ROIs) over time with different treatment schemes. All intensity values for each ROI were normalized to their initial value for analysis (2 min after FITC-dextran injection).

Inhibition of leukocyte–endothelial interaction

Rat anti-mouse selectin monoclonal antibodies MEL-14 (anti-L-selectin; rat IgG2a k, Becton-Dickinson, USA) 0.1 mg, monoclonal antibodies RB40.34 (anti-P-selectin; rat IgG1λ, Becton Dickinson) 0.1 mg and monoclonal antibodies 10E9.6 (anti-E-selectin; rat IgG2a k, Becton Dickinson) 0.1 mg were mixed and administered intraperitoneally for functional blocking 1 h before PDT. The selectivity and efficiency of these antibodies were previously validated by our group and others [13–15].

Assessment of the leukocyte–endothelial interaction

Leukocyte–endothelial interaction was assessed before, 1 and 2 h after PDT. To perform this, 100 μl of rhodamine-6-G (0.05%, Sigma-Aldrich, Buchs, Switzerland) was injected intravenously to stain leukocytes. Because of the high mitochondrial content of leukocytes, rhodamine-6-G preferentially accumulates in the cytoplasm of these cells. For each time point of interest, image fields were recorded on a videotape for 1 min that were then analysed offline.

The rolling of leukocytes was determined by counting the number of rolling cells passing a 100 μm vessel segment during a 30 s time frame. Results were expressed as the number of cells per cross-section circumference of vessel segments (cells/mm/30 s). Leukocytes were considered as rolling when their velocity along the vessel wall was lower than those of erythrocytes [16].

Recruitment of leukocyte in a PDT-treated site was assessed and expressed as the number of cells per surface area (cells/mm^2). Leukocytes were considered as recruited when they remained stationary in the treated zone for a minimum of 30 s.

Statistical analysis

To analyse the variables 'rolling' and 'recruitment', we applied the analysis of variance (ANOVA) test with repeated measures (time as within factor and group as between factor) using the software Statistica 9. The variable 'recruitment' was transformed before analysis in order to satisfy the assumptions of ANOVA with repeated measures application (log transformation). Tukey's honestly significant difference *post hoc* test was run for multiple comparisons. To analyse the integrated fluorescence inside tumour or the difference between tumour and normal tissues, multilevel models with 'time' and 'group' as fixed effects and 'mice' as random effect were created. A weight on 'group' was added in order to modelize the heteroscedasticity due to groups. The outcome was transformed before analysis in order to satisfy the assumptions of multilevel application (square root transformation). The package 'NLME' of the software R was used for the analysis. For *post hoc* multiple comparison, Bonferroni corrections were used. Data were expressed as mean ± standard error of mean. Statistical significance was accepted at $P < 0.05$.

RESULTS

To assess the permeability of tumour microvessels and leukocyte behaviour under different treatment conditions, we grew human mesothelioma xenografts (H-meso-1) in nude mice chronically instrumented with dorsal skinfold chambers (Fig. 1). This model allowed real-time and sequential monitoring of these parameters for up to 3 weeks after tumour implantation.

A representative real-time assessment of FITC-dextran leakage in tumour tissue following PDT is shown in Fig. 2. FITC-dextran extravasation was then quantified in tumour and normal tissues. PDT significantly enhanced FITC-dextran extravasation in tumour but not in normal tissue, applying the same drug and light dose (Fig. 3).

We then assessed leukocyte–endothelial interaction. A typical real-time image of rhodamine-labelled leukocyte recruitment

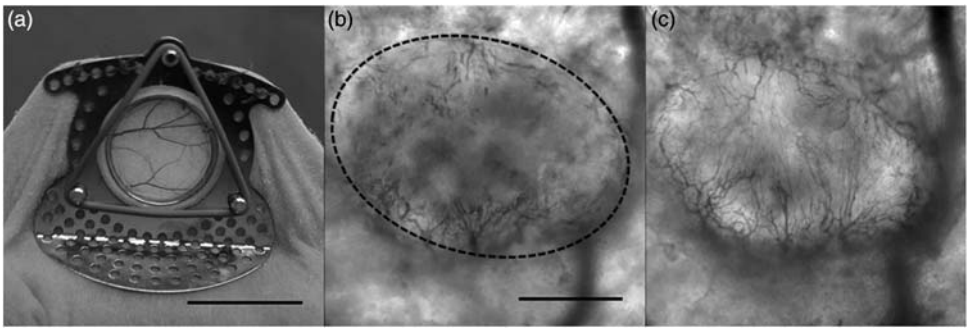

Figure 1: (**a**) Skinfold chamber preparation in nude mice (bar: 10 mm). (**b**) Partially vascularized human malignant mesothelioma xenograft (circle, dotted line) 7 days after transplantation into the skinfold chamber (intravital imaging, objective ×4, length of bar: 1 mm). (**c**) Fully vascularized mesothelioma xenograft 14 days after implantation.

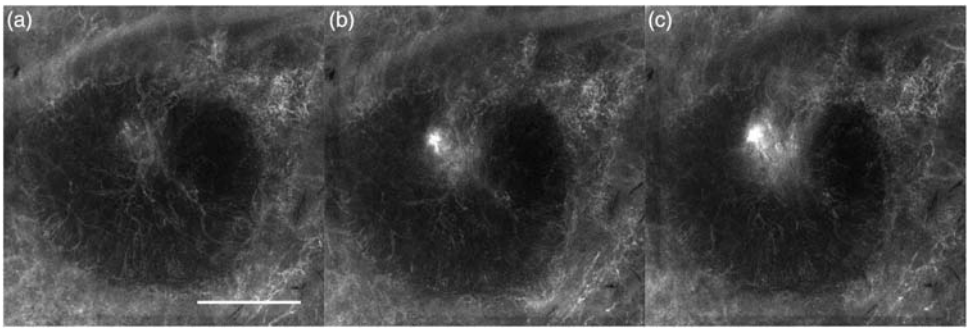

Figure 2: Typical example of PDT-induced FITC-dextran (MW 2000 kDa) leakage to the mesothelioma xenograft (**a**) 2.5 min, (**b**) 20 min and (**c**) 45 min time point after systemic drug injection; length of bar: 1 mm.

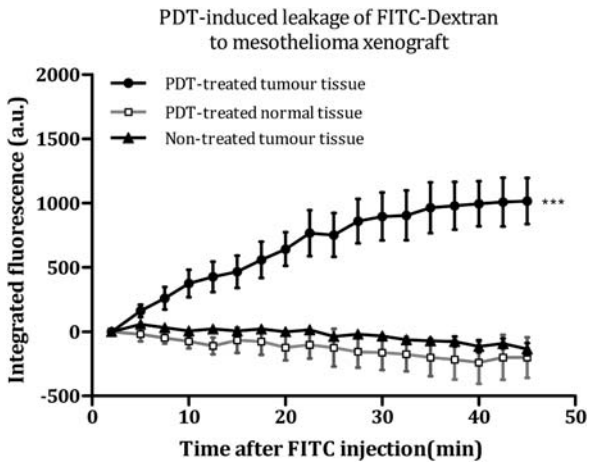

Figure 3: PDT-induced leakage recorded by the time-dependent evolution of the FITC-dextran-integrated fluorescence in the dorsal skinfold chamber model. Seven minutes after FITC-dextran injection, PDT induced a significantly enhanced extravasation of FITC-dextran in the PDT group ($P < 0.05$). The human mesothelioma xenograft and normal tissue were both treated with a light dose of 60 J/cm^2, 10 min after tail vein injection of 0.4 mg/kg of Visudyne. Data are presented as mean ± SEM. ***$P < 0.001$ when compared with PDT-treated normal tissue and non-treated tumour tissue.

following PDT is shown in Fig. 4. Leukocyte rolling and recruitment were quantified in different treatment groups: control, PDT, PDT plus isotype antibodies (isotype group) and PDT plus a functionally blocking anti-pan-selectin antibody cocktail (MABS group) (Figs 5 and 6). PDT and PDT plus the isotype antibody significantly increased the mean number of rolling leukocytes and their recruitment to the PDT-treated area in tumours up to 2 h following therapy (Figs 5 and 6). In contrast, the anti-pan-selectin antibody cocktail significantly attenuated PDT-induced leukocyte rolling and recruitment in tumour vessels. To assess whether leukocyte–endothelial interaction affected drug distribution, we compared the extravasation of FITC-dextran in tumours treated by PDT plus the anti-pan-selectin antibody cocktail with the other treatment groups. FITC-dextran extravasation was significantly enhanced in all PDT treatment groups, irrespective of whether the isotype or the functionally blocking anti-pan-selectin antibody cocktail was given (Fig. 7).

DISCUSSION

In the current study, we assess the use of low-level PDT for targeted drug delivery to MPM. PDT-mediated drug delivery works by induction of an increased transendothelial transfer of circulating intravascular drugs to the tumour interstitium, resulting in a preferential accumulation of an intravenously administered drug in the irradiated area. While the PDT itself has most probably no tumouricidal activity and no toxicity in this context, the treatment effect is achieved by the increased uptake of the circulating drug (e.g. chemotherapy and immunotherapy) in the PDT-treated area [9, 17].

The principal finding of this experimental study is that low-dose PDT leads to a robust and selective uptake of a circulating macromolecular drug in human malignant mesothelioma xenografts, but not in normal tissue.

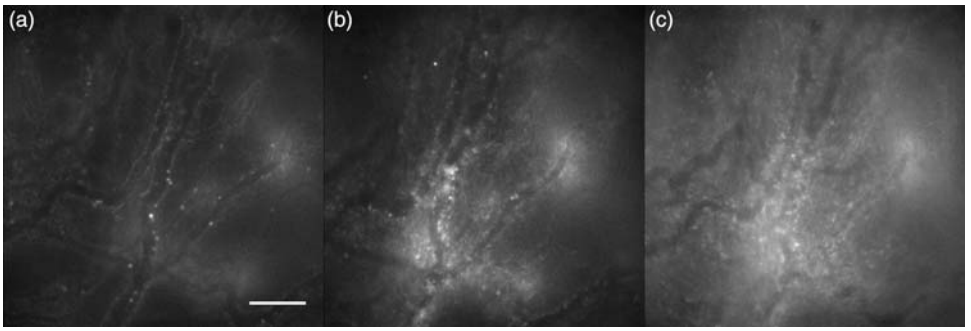

Figure 4: Typical example of leukocyte recruitment after PDT in the illuminated area of the mesothelioma xenograft: (**a**) before, (**b**) 1 h and (**c**) 2 h after treatment; length of bar: 100 μm.

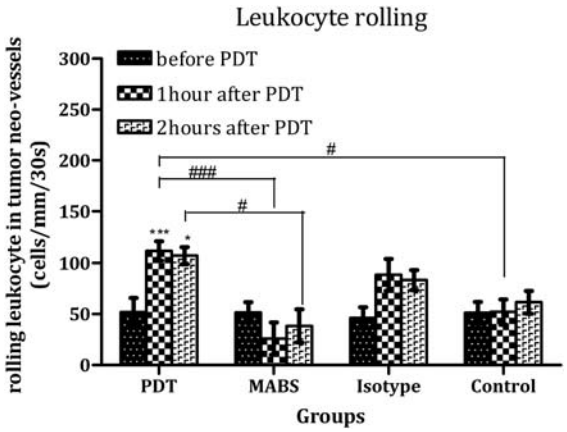

Figure 5: Rolling of leukocytes was measured before and 1 and 2 h after PDT in the neovasculature of human mesothelioma xenografts. Data are presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{*}P < 0.05$ and $^{***}P < 0.001$ when compared with the baseline (before PDT) value in each group.

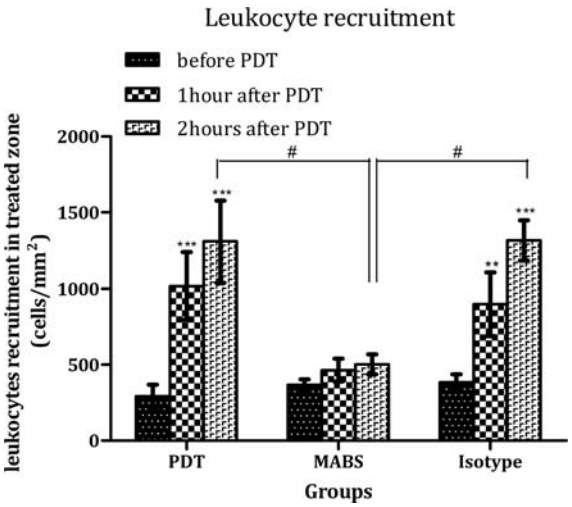

Figure 6: Leukocyte recruitment was measured before and 1 and 2 h after PDT in the human mesothelioma xenograft-treated site of various groups. Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with the baseline value (before PDT) in each group. $^{\#}P < 0.05$.

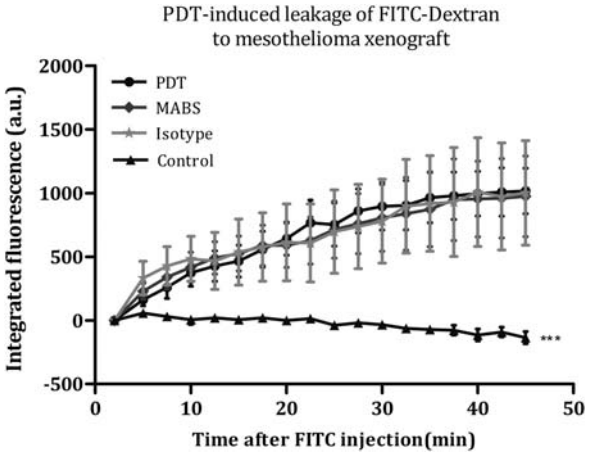

Figure 7: Effect of leukocyte–endothelial interaction modulation on the PDT-mediated extravasation of FITC-dextran in human mesothelioma xenografts. PDT was performed at a light dose of 60 J/cm^2 10 min after intravenous injection of 0.4 mg/kg Visudyne, followed by intravenous administration of FITC-dextran 1 h after PDT. Animals in the MABS group received specific antibodies against selectins, and animals in the isotype group received non-specific antibodies. Control animals had Visudyne administration but no irradiation or antibodies. Bars indicate the SEM. $^{***}P < 0.001$ when compared with PDT, MABS and isotype groups.

The macromolecular model drug (FITC-dextran, MW 2000 kDa) was chosen in this study to reflect the dimensions of modern cytostatic compounds such as liposomal formulation of doxorubicin or cisplatin [18]. Due to the fluorescent label, we could document by intravital fluorescence microscopy (i) its intravascular distribution after intravenous injection, (ii) the transfer from the intravascular to the extravascular space and (iii) tissue accumulation over time (Fig. 2).

During the observation period of 45 min, we observed a roughly 1000-fold increase (arbitrary units, normalized data) in the intensity of the fluorescent signal within the PDT-treated zone of the mesothelioma xenografts, corresponding to a robust extravasation and tissue accumulation of the macromolecular drug in the tumour tissue (Fig. 3). No spontaneous uptake of FITC-dextran in untreated tumour tissue occurred during the observation period, demonstrating the well-known difficulty of drug delivery to tumours [19]. Most importantly, PDT on normal tissue (striated muscle and fat tissue) under the same treatment conditions had no effect on extravasation of FITC-dextran at all, with a fluorescent signal decreasing over time, due to metabolism and excretion (Fig. 3).

We can only speculate about the reasons for this remarkable selectivity for tumour tissue. First, the minimally required photosensitizer and light doses to induce efficient drug extravasation

were found to be much lower in tumour than in normal tissue. In our recent study on the same animal model, in which we studied PDT-induced drug delivery to 'non-malignant' tissues [8], we needed to apply a fluence of 200 J/cm^2 and a Visudyne dose of 800 μg/kg b.w. for efficient extravasation of FITC-dextran. In contrast, we have now applied half of the drug dose and less than a third of the light dose (60 J/cm^2) in order to induce drug accumulation in mesothelioma xenografts, hence well below the threshold dose for normal tissues. It is well known that the vascular effect of PDT is dose-dependent [20], ranging from vasoconstriction and reversible endothelial damage at low doses, interrupted flow and transient thrombosis at intermediate doses, to irreversible vessel occlusion at highest PDT doses. It has also been well documented that newly formed tumour neovessels are less resistant to the applied PDT dose than mature vessels in normal tissue [21]. Conversely, high PDT doses would interfere with the tumour uptake of a circulating drug. In the light of the differential response of tumour and normal vessels to comparable PDT doses [21], it is not unreasonable to warrant that the microcirculation in tumour neovessels would be actively interrupted at a high PDT dose which would enhance transendothelial permeability in normal mature vessels. In such a scenario, the microcirculatory impairment would then shield the irradiated tumour from efficient drug delivery, and the opposite of the desired effect would be seen. In this respect, the effects observed in our study provide an important experimental proof of principle.

However, it could also be speculated that the selective effect of PDT-induced drug delivery to malignant mesothelioma tissue is due to soluble hyperpermeability mediators affecting the endothelial barrier, such as vascular endothelial growth factor (VEGF). VEGF is known to be induced by PDT under certain conditions in tumours, but only to a lesser degree in normal tissue [22].

Leukocytes are known as important effectors of microvessel permeability [23]. We hypothesized that the observed effect of PDT on the permeability in neovessels of mesothelioma xenografts may be explained by activation of the adhesion cascade, as observed in response to other inflammatory stimuli [14]. Activated leukocytes induce increased microvascular vessel permeability by enzyme release and oxidative burst when interacting with the microvessel walls. Activation of leukocyte requires their rolling and adhesion, a process which depends on different subtypes of selectins. Indeed, in a recent study, we have shown that PDT renders microvessel walls in 'non-malignant' tissue more permeable to water and macromolecular drugs through a leukocyte-mediated mechanism. Suppression of leukocyte–endothelial interaction diminished the PDT-induced drug leakage in normal tissue by 70% [8]. It is hence a surprise to note that in the malignant mesothelioma, leukocyte–endothelial cell interaction seems to play no pathomechanistic role. To assess whether interaction between tumour vessel endothelium and leukocytes is the critical event leading to permeability enhancement, we suppressed in the current study the leukocyte adhesion by systemic administration of functionally blocking antibodies directed against selectins. Without antibodies or with non-specific antibodies, PDT induced a strong inflammatory tissue reaction in the PDT-treated zone of the mesothelioma xenograft, as documented by *in vivo* observation of significantly increased leukocyte rolling along the endothelium, transmigration and recruitment in the interstitium for up to 2 h of observation (Figs 5 and 6). This is in accordance with results of other groups [24]. As expected, the anti-adhesion treatment by pan-selectin antibodies led to a significant reduction in the inflammatory reaction (Figs 5 and 6). However, this inhibition of leukocyte adhesion had no influence on PDT-induced drug delivery to mesothelioma xenografts (Fig. 7). PDT led to the targeted transfer of FITC-dextran from the intravascular to the interstitial compartment in the irradiated zone of the mesothelioma xenograft, whether or not the leukocyte cascade was inhibited. Apparently, PDT-related actions other than the inflammatory tissue response must be responsible for the observed phenomenon of PDT-induced drug delivery. Tumour vasculature is structurally and functionally abnormal. Tumour vessels are innately leaky and show morphological and functional abnormalities and an incomplete basement membrane [19]. The leakiness of tumour vessels results in an increased interstitial fluid pressure, forming a barrier to transcapillary transport and efficient delivery of therapeutic agents [20]. Interestingly, there is evidence in the literature that PDT has the capacity to temporarily reduce the interstitial fluid pressure, presumably due to transitory vasoconstriction and reduced blood flow [25]. Since macromolecular drugs such as FITC-dextran are mainly transported from the intravascular compartment to the tumour interstitium by convection (and not by diffusion), their transport depends on the transendothelial flow of fluid (and not on the difference of their concentration). In consequence, it is reasonable to speculate that the PDT-induced drug delivery in tumours is likely not induced by alteration of the endothelial barrier, which is already disturbed at baseline, but rather by modification of the transendothelial flow.

In conclusion, low-dose PDT enhances targeted delivery of a macromolecular drug to human mesothelioma xenografts in a tumour-selective way. This observation provides a valuable proof of principle and warrants further studies, eventually aiming at novel therapy concepts of mesothelioma patients. Intracavitary low-dose PDT after pleuropneumonectomy could enhance the uptake of concomitant systemically administered cytostatic agents in remnant tumour islets and thereby improve local control.

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Conflict of interest: none declared.

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