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Distribution of Free and Liposomal Doxorubicin After Isolated Lung Perfusion in a Sarcoma Model

THESE

préparée sous la direction du Professeur Hans-Beat Ris
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Rapport de synthèse

DISTRIBUTION DE LA DOXORUBICINE LIBRE ET LIPOSOMALE APRES PERFUSION ISOLEE DU POUMON DANS UN MODELE SARCOMATEUX

Introduction

La perfusion isolée du poumon à l'aide de Doxorubicine libre et une nouvelle forme de Doxorubicine liposomale pégylée (Liporubicine) est comparé en terme de pénétration et accumulation de Doxorubicine dans le tissu tumoral et pulmonaire dans un modèle de rats porteurs de tumeur sarcomateuse au niveau du poumon gauche.

Matériel et méthode

Une tumeur sarcomateuse unique a été générée dans le poumon gauche de 39 Fischer rats, suivi 10 jours plus tard, par une perfusion isolée du poumon gauche (n =36) avec Doxorubicine libre (n=18) et Liporubicine (n=18) à une dose de 100 µg (n=9) et 400 µg (n=9) pour chaque formulation de Doxorubicine. Dans chaque poumon perfusé, la concentration de l'agent cytostatique et sa distribution ont été investiguées dans la tumeur et trois parties du poumon normal par HLPC (n=6) et par microscopie de florescence (n=3). Des analyses histologiques et immunohistochimiques (facteur von Willebrand) ont été effectuées sur trois animaux non traités.

Résultats

Les tumeurs sarcomateuses dans les animaux de contrôle démontraient une bonne vascularisation avec de fines branches capillaires qui étaient présentes partout dans les tumeurs. La perfusion isolée du poumon démontrait une distribution de l'agent cytostatique d'une manière hétérogène dans le poumon perfusé et une concentration de Doxorubicine inférieure dans les tumeurs par rapport au tissu pulmonaire sein pour les deux formulations de Doxorubicine et les deux doses appliquées. La perfusion isolée du poumon avec Doxorubicine libre démontrait une concentration significativement plus élevée que Liporubicine dans la tumeur et le parenchyme pulmonaire pour les deux doses appliquées ($p < 0,01$). Néanmoins, le coefficient de concentration tumorale et pulmonaire était plus bas pour Doxorubicine libre que pour Liporubicine pour une dose de 100 µg (0.27 ± 0.1 vs 0.53 ± 0.5, $p=0.23$) tandis qu'il était similaire pour les deux formulations de Doxorubicine à une dose de 400 µg (0.67 ± 0.2 vs 0.54 ± 0.2, $p=0.34$). Les deux formulations de Doxorubicine émergeaient un signal de fluorescence provenant de tous les compartiments du parenchyme pulmonaire mais seulement un signal sporadique et faible émergeant des tumeurs, provenant de la périphérie de la tumeur et des vaisseaux situés à l'intérieur de la tumeur, pour les deux doses appliquées.

Conclusion

La perfusion isolée du poumon démontrait une distribution hétérogène de la Doxorubicine et sa forme liposomale dans le poumon perfusé et une accumulation plus faible dans la tumeur que dans le tissu parenchymateux adjacent pour les deux formulations de Doxorubicine et les deux doses appliquées.

Distribution of Free and Liposomal Doxorubicin After Isolated Lung Perfusion in a Sarcoma Model

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Background. Isolated lung perfusion (ILP) with free and a novel liposomal-encapsulated doxorubicin (Liporubicin, CT Sciences SA, Lausanne, Switzerland) was compared with respect to drug uptake and distribution in rat lungs bearing a sarcomatous tumor.

Methods. A single sarcomatous tumor was generated in the left lung of 39 Fischer rats, followed 10 days later by left-sided ILP (n = 36) with free and equimolar-dosed liposomal doxorubicin at doses of 100 μg (n = 9) and 400 μg (n = 9) for each doxorubicin formulation. In each perfused lung, the drug concentration and distribution were assessed in the tumor and in three areas of normal lung parenchyma by high-performance liquid chromatography (n = 6) and fluorescence microscopy (n = 3). Histologic assessment and immunostaining with von Willebrand factor was performed in 3 animals with untreated tumors.

Results. The sarcomatous tumors in controls were well vascularized with fine branching capillaries present throughout the tumors. Isolated lung perfusion resulted in a heterogeneous drug distribution within the perfused lung and a consistently lower drug uptake in tumors than in lung parenchyma for both doxorubicin formulations and both drug doses applied. Isolated lung perfusion with free doxorubicin resulted in a significantly higher drug uptake than Liporubicin in both the tumor and lung tissue for both drug doses applied ($p < 0.01$). However, the tumor/normal tissue drug ratio was lower for free than for liposomal doxorubicin at a drug dose of 100 μg (0.27 ± 0.1 vs 0.53 ± 0.5; $p = 0.225$) and similar for both doxorubicin formulations at a drug dose of 400 μg (0.67 ± 0.2 vs 0.54 ± 0.2; $p = 0.335$). Both doxorubicin formulations resulted in fluorescence signaling emerging from all tissue compartments of normal lung parenchyma but only in weak and sporadic signaling from the tumors confined to the tumor periphery and vessels situated within the tumor for both drug doses assessed.

Conclusions. Isolated lung perfusion with free and liposomal doxorubicin resulted in a heterogeneous drug distribution within the perfused lung and in a lower drug uptake in tumors than in lung tissue for both doxorubicin formulations and drug doses applied.

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Isolated lung perfusion (ILP) of cytostatic drugs has been assessed under clinical [1–8] and experimental [9–15] conditions for various agents, including doxorubicin [1–3, 10–14, 16] , melphalan [8], platinum [4, 7], and tumor necrosis factor-α (TNF-α) combined with interferon-γ [5]. It allows for a targeted delivery of the drug to the lung with an up to sevenfold higher drug concentration in lung tissue. Plasma levels after ILP are significantly lower compared with intravenous application of the cytostatic drug [13, 14]. Under clinical conditions, however, ILP has failed to demonstrate a relevant antitumor activity, irrespective of the cytostatic agent administered [1–5, 7].

Doxorubicin, an active anthracycline against sarcoma, has been extensively tested in ILP [15]. Doxorubicin-based ILP in sarcoma-bearing rats resulted in a high drug uptake in lung tissue, minimal systemic toxicity, and measurable antitumor activity [10–12]. However, clinical studies suggest a lower drug uptake in tumors than in the surrounding normal lung tissue after doxorubicin-based ILP [1–3]. In addition, experimental doxorubicin-based ILP in a porcine model has shown a heterogeneous pattern of drug distribution within the perfused lung despite a high overall lung drug uptake [16].

Liporubicin (CT Sciences SA, Lausanne, Switzerland) is a newly developed pegylated, liposomal-encapsulated formulation of doxorubicin. Liposomal-encapsulated drugs have been shown to evade the immune system, and their surface features promote increased permeability of the tumor vasculature for these compounds, leading to a preferential accumulation within tumor tissues [17–20]. We hypothesized that Liporubicin-based ILP may result in a better tumor drug uptake and in an enhanced ratio of tumor/normal lung tissue concentration compared with free doxorubicin. For this purpose, ILP with two doses of free doxorubicin and equimolar-dosed Liporubicin were compared in a sarcoma model

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with respect to the concentration and distribution of the cytostatic agent in the tumor and normal lung tissue.

Material and Methods

Study Design

Single-pass gravity-driven antegrade ILP of the left lung was performed in 39 animals bearing a sarcoma tumor in their left lung that was generated by a subpleural injection of a sarcoma cell suspension 10 days before ILP, as previously described [21]. Nine animals were perfused with 100 μg doxorubicin and 9 with an equimolar dose of Liporubicin; 9 animals were perfused with 400 μg doxorubicin and 9 with an equimolar dose of Liporubicin. Sixty minutes after restoration of the pulmonary circulation, the animals were sacrificed and the perfused lungs were harvested. The doxorubicin concentration and distribution in the tumor and surrounding lung were assessed by high-performance liquid chromatography (HPLC) in 6 animals of each group and by fluorescence microscopy for 3 animals of each group. The tumors of 3 untreated animals underwent histologic assessment and immunostaining with von Willebrand factor to assess their vascularization.

Animals and Housing

Male Fischer 344 rats, weighing 250 to 300 g (Charles River, L'Arbresle, France) were used. They had free access to standard laboratory rat chow and water and were housed with a 12:12 hour light-dark circle under controlled temperature. They were treated in accordance with the Animal Welfare Act, the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, and according to the Local Ethical Committee of the University of Lausanne.

Tumor Cell Line

A syngeneic methylcholanthrene-induced sarcoma (MCA) cell line kindly supplied by the Memorial Sloan-Kettering Cancer Center (New York, NY) was used. The MCA cell line was cultivated at 37°C and 5% carbon dioxide in 20 mL of Roswell Park Memorial Institute (RPMI)-1640 medium containing glutaryl, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Invitrogen Corp, GIBCO Life Technologies Ltd, Paisley, United Kingdom). For the preparation of the cell suspension, the cells were washed twice with phosphate-buffered saline (PBS) and detached with 4 mL of trypsin. Tumor cell vitality was assessed in a hematocytometer after centrifugation at 1000*g* for 4 minutes, washing, resuspension in PBS, and addition of trypan blue. The cell suspension was adjusted to a density of 5.0×10^7 vital cells/mL.

Generation of Sarcoma Tumors Within the Left Lung

Anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and 0.1% atropine (0.05 mL/kg). Orotracheal intubation was performed by tracheal insertion of a 16-gauge polyethylene angiocatheter (Becton Dickinson, Sandy, UT) using a laryngoscope. The catheter was connected to a standard rodent ventilator (Model 683, Harvard Apparatus Inc, Les Ulis, France) and ventilation was performed with a mixture of oxygen and 0.5% to 2% isoflurane (Forene, Abbott, Baar, Switzerland). A tidal volume of 10 mL/kg was adjusted with a respiratory rate of 75 to 90 breaths/min. A small left-sided thoracotomy was performed through the seventh intercostal space, and 0.1 mL of MCA cell solution containing 5.0×10^6 viable tumor cells were injected subpleural into the left lower lobe by use of a 27-gauge needle and a 0.3-mL insulin syringe [21]. The thoracotomy was closed in layers, and the endotracheal tube was removed after spontaneous breathing of the animals.

Antegrade Isolated Perfusion of the Left Tumor-Bearing Lung

Ten days after tumor implantation, single-pass antegrade ILP was performed in 36 animals. Eighteen animals were perfused with doxorubicin (Adriblastin, Pharmacia & Upjohn, Dübendorf, Switzerland) and 18 with Liporubicin.

The animals were anesthetized as described. A left-sided thoracotomy was performed through the fourth intercostal space. An operative microscope was used (original magnification ×16; Carl Zeiss, Jena, Germany). The inferior pulmonary ligament was divided, and the left lung was retracted anteriorly. The mediastinal pleura were opened, and the left phrenic nerve was preserved.

The left pulmonary artery and vein were dissected. The vessels were encircled with 8-0 Prolene (Ethicon, Somerville, NJ) suture and clamped. Small transverse incisions were performed in the PA and PV distal to the clamps using microvascular scissors, and both vessels were cannulated by use of 2 catheters consisting of a 2-cm polyethylene tube (0.10 mm internal diameter, 0.30 mm outer diameter) connected to 20-cm polyethylene tube (0.30 mm internal diameter, 0.60 mm outer diameter; Clinical Plastic Products SA, La Chaux-de-Fonds, Switzerland).

Single-pass antegrade ILP with doxorubicin or equimolar dosed Liporubicin dissolved in 5 mL of 6% hydroxyethyl starch (HAES; Intramed, Woodmead, Sandton, South Africa), was performed for 20 minutes using a roller pump at a flow-rate of 0.25 mL/min. Antegrade ILP consisted of drug administration through the pulmonary artery and effluent collection from the pulmonary vein.

The cytostatic perfusion was followed by a washout phase of the perfused isolated lung for 10 minutes with 5 mL of 6% HAES at the same perfusion conditions. The lung was ventilated during ILP with a positive end-expiratory pressure (PEEP) of 2 to 3 cm H_2O. After completion of ILP, the catheters were removed, and the vessels were repaired by interrupted transverse sutures using 10-0 Prolene, followed by declamping and restoration of the circulation. Ventilation with a PEEP of 2 to 3 cm H_2O was continued for 60 minutes after restoration of the circulation. At this point, the animals were sacrificed and the perfused lung was harvested.

Assessment of Doxorubicin Concentration in Tumor and Lung Tissues

After the lungs were harvested, the tumor nodule was dissected from the surrounding normal lung tissue by use of the operation microscope, and tumor and normal tissue of the perfused lung were analyzed separately. After dissection and excision of the tumor, the tumor-free perfused lung tissue was cut in 3 pieces corresponding to the upper, middle, and lower part of the lung. The samples were stored at −80°C before analysis. Doxorubicin concentration measurements in the tumor and lung tissues were performed by HPLC as previously described [22]. For Liporubicin concentration measurements, the technique has been adjusted for the release of doxorubicin from liposomes, in accordance with Chin and colleagues [23].

The concentrations of doxorubicin in tissues were separately determined in the upper, middle, and lower part of the tumor-free parenchyma of each perfused lung to assess the spatial drug distribution within the perfused lung. Coefficients of variation were calculated from these three values for each perfused lung as previously described [16].

Assessment of Drug Distribution by Fluorescence Microscopy

The perfused lungs were harvested, snap frozen in liquid nitrogen by contact with isopentane slush, stored at −70°C, and processed for fluorescence microscopy as previously described [24]. The frozen tissue blocks were mounted in optimal cutting temperature medium (Tissue-Tek II embedding compound, Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and a series of sections was cut with a cryomicrotome (Frigocut Model 2700, Reichert, Switzerland.).

Six consecutive, nonstained 5-μm-thick tissue sections mounted on clean glass slides were prepared for each sample. From each frozen section, three images were recorded over three different parts of the slice to avoid photo bleaching. We used a Zeiss AxioPlan-2 microscope equipped with a Zeiss Axiophot image analysis system (Carl Zeiss) and a filtered 100-W mercury lamp as excitation light source. Images were recorded with a grey-scale camera with a 12-bits dynamic range and 2 × 2 binning, resulting in 694- × 520-pixel images with 4095 grey levels. A filter "cube" (set 40012, Chroma Technology Corp, Rockingham, VT; excitation: HQ 480/40x, dichotic mirror: Q505LP) and a barrier filter (HQ510LP) have been used for epifluorescence measurements.

After recording the fluorescence images, the same slices were stained with hematoxylin and eosin. A hematoxylin and eosin image was recorded from the identical position and compared with the fluorescence image to determine the histologic localization of doxorubicin using the public domain ImageJ 1.31 program (National Institutes of Health, Bethesda, MD) [25]. This technique allows the detection of the fluorescence signaling of doxorubicin in different tissue compartments of normal lung parenchyma and within the tumor.

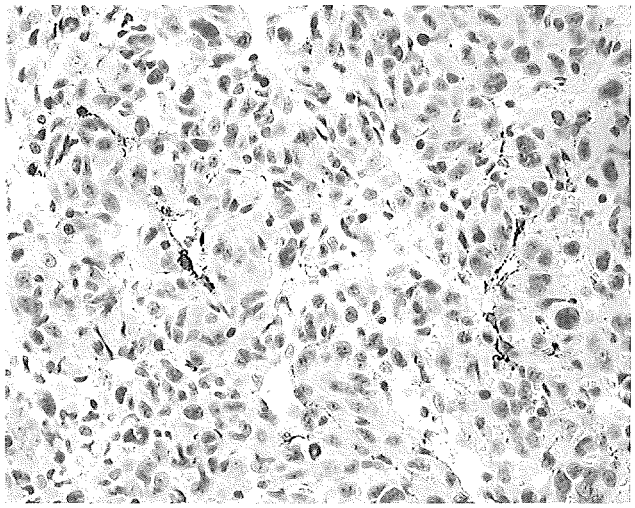

Fig 1. Immunostaining with von Willebrand factor of untreated sarcoma tumors reveals a well-circumscribed sarcomatous tumor mainly formed by large undifferentiated cells with a vascular network consisting of fine branching capillaries present throughout the tumor. Immunostaining highlights endothelial cells and the vascular bed in the tumors (original magnification × 400).

Statistical Analysis

Doxorubicin concentrations in tumors and normal lung parenchyma were compared for both doxorubicin formulations using a Student *t* test for unrelated samples. Variabilities in doxorubicin lung tissue levels were expressed as the coefficients of variation of tissue concentration in the three parts of perfused lungs for both doxorubicin formulations, and drug doses and were compared using the Student *t* test. A bidirectional hypothesis was applied and significance accepted at $p < 0.05$.

Results

Controls

Histologic assessment of 3 untreated tumors revealed a well-circumscribed sarcomatous tumor in each lung, mainly formed by large undifferentiated cells. Immunostaining by von Willebrand factor revealed an extensive vascular network composed of small vessels and capillaries present throughout the tumors (Fig 1). The tumor size ranged from 3 to 8 mm in diameter. The mitotic index ranged from 12 to more than 100 mitoses per 10 high power fields (surface, 0.221 mm^3/ field). Spontaneous necrosis was observed in less than 2% of the tumor volume for each case analyzed. No tumor growth within the pleural cavity was identified in any of the animals.

Isolated Lung Perfusion

Thirty-five animals underwent successful antegrade left ILP with doxorubicin and Liporubicin, respectively. One animal had to be excluded from concentration measurements of free doxorubicin because of leakage and insufficient drug exposure of the lung during perfusion.

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After the lungs were harvested, the tumor nodule was dissected from the surrounding normal lung tissue by use of the operation microscope, and tumor and normal tissue of the perfused lung were analyzed separately. After dissection and excision of the tumor, the tumor-free perfused lung tissue was cut in 3 pieces corresponding to the upper, middle, and lower part of the lung. The samples were stored at −80°C before analysis. Doxorubicin concentration measurements in the tumor and lung tissues were performed by HPLC as previously described [22]. For Liporubicin concentration measurements, the technique has been adjusted for the release of doxorubicin from liposomes, in accordance with Chin and colleagues [23].

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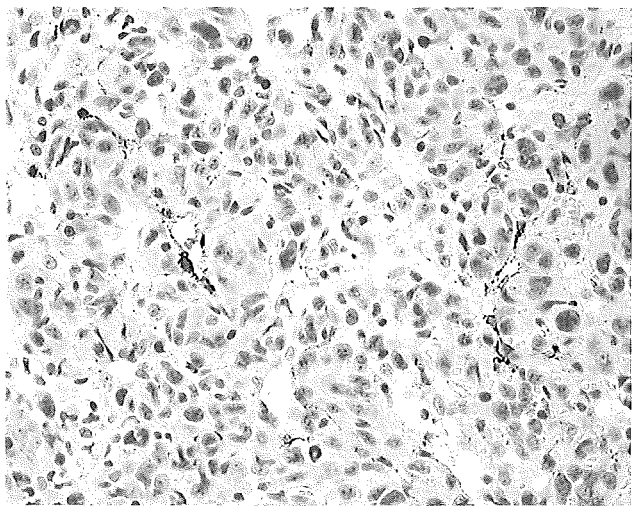

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Table 1. Results of Isolated Lung Perfusion With Free Doxorubicin and Equimolar-Dosed Liporubicin

	Drug Dose, 100 μg		Drug Dose, 400 μg	
Result	Free Doxorubicin	Liporubicin	Free Doxorubicin	Liporubicin
Tissue concentration[a]				
Lung, μg/g	13.8 ± 4.3	2.0 ± 0.7	58.5 ± 20.1	5.2 ± 3.7
Tumor, μg/g	3.9 ± 2.5	0.8 ± 0.5	36.9 ± 10.4	3.2 ± 3.5
Ratio[b]	0.27 ± 0.1	0.53 ± 0.5	0.67 ± 0.2	0.54 ± 0.2
CV%[c]	27%	49%	24%	28%

[a] These data are presented as the mean ± standard deviation. [b] Ratio of tumor/normal tissue drug concentrations. [c] CV is the coefficient of variation of doxorubicin levels measured in the upper, middle, and lower tumor-free part of the perfused lung.

Doxorubicin Concentration in Tumor and Normal Lung Parenchyma After Isolated Lung Perfusion

Table 1 reports the drug concentrations in the tumor and lung parenchyma after ILP for both doxorubicin formulations and both drug doses applied as well as the coefficient of variation (CV%) of drug tissue concentrations in the upper, middle, and lower parts of the perfused lungs.

Isolated lung perfusion led to a heterogeneous spatial drug distribution within the perfused lung for both doxorubicin formulations and both drug doses as demonstrated by the large coefficients of variation. In addition, there was a wide interanimal variability of drug concentrations in the tumor and normal lung tissue for both doxorubicin formulations.

FREE DOXORUBICIN. Increasing the administered dose from 100 μg to 400 μg resulted in a fourfold increased drug uptake in lung tissue (13.8 vs 58.5 μg/g; $p = 0.001$) and in an approximately tenfold increased uptake in tumors (3.9 vs 36.9 μg/g; $p = 0.0001$). Accordingly, this significantly improved the tumor/normal tissue drug ratio from 0.27 ± 0.1 to 0.67 ± 0.2 ($p = 0.003$). However, the doxorubicin concentrations in tumors were always lower than in lung tissue for both drug doses and all animals assessed.

LIPORUBICIN. Increasing the equimolar-dosed doxorubicin dose from 100 μg to 400 μg resulted in an almost threefold higher drug uptake in lung tissue (2.0 vs 5.2 μg/g) and a fourfold higher uptake in tumors (0.8 vs 3.2 μg/g). However, due to the large interanimal variability, this difference was not statistically significant. Both drug doses resulted in a similar tumor/normal tissue drug ratio of 0.53 ± 0.5 vs 0.54 ± 0.2 ($p = 1.0$). The drug concentrations were always lower in the tumor than in lung tissue for both drug doses and in all but 1 animal assessed.

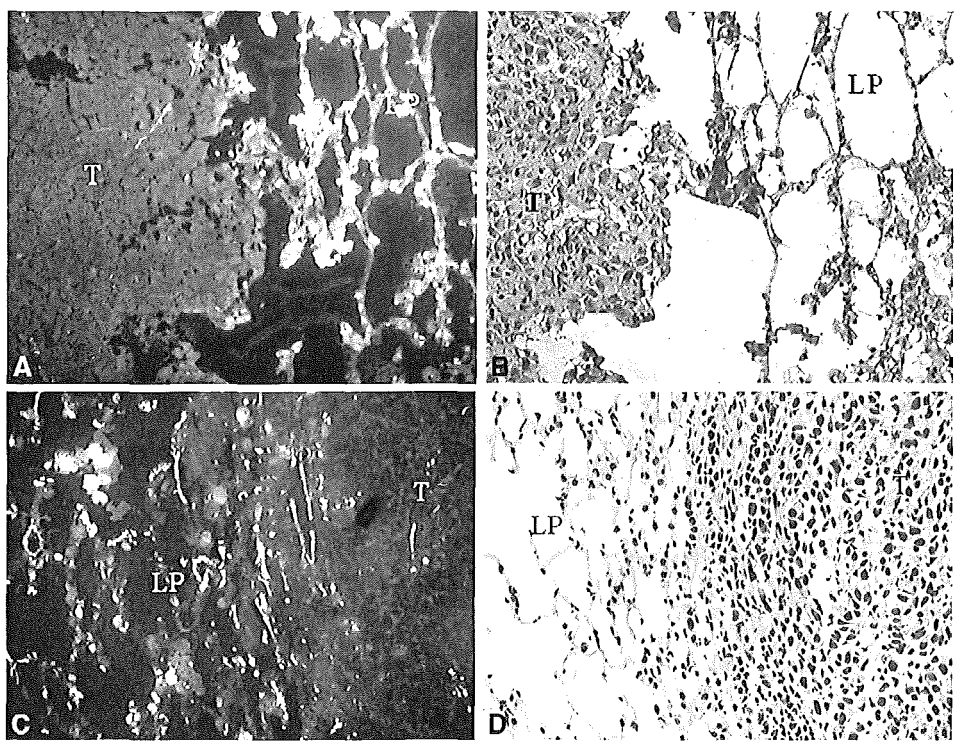

Fig 2. Fluorescence microscopic assessment of the perfused lungs after isolated lung perfusion (ILP) at a drug dose of 100 μg. (A) Fluorescence photomicrograph and (B) corresponding hematoxylin and eosin, after doxorubicin-based ILP reveal weak and sporadic signaling confined to the tumor (T) periphery and surrounding lung parenchyma (LP). (C) Fluorescence photomicrograph and (D) corresponding hematoxylin and eosin stain, after Liporubicin-based ILP reveal weak and sporadic signaling confined to the tumor periphery and surrounding lung parenchyma (original magnification × 40).

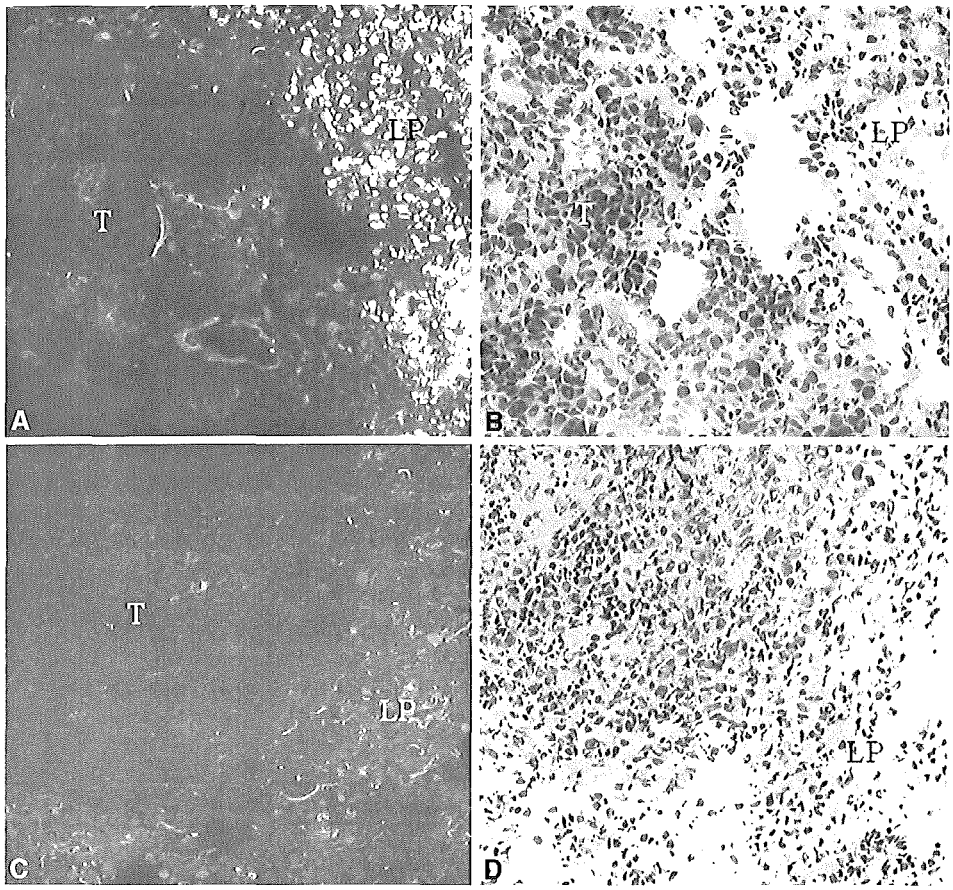

Fig 3. Fluorescence microscopic assessment of the perfused lungs after isolated lung perfusion (ILP) at a drug dose of 400 μg. (A) A fluorescence photomicrograph and (B) a corresponding hematoxylin and eosin stain after doxorubicin-based ILP reveal weak and sporadic signaling confined to the tumor (T) periphery and surrounding lung parenchyma (LP). (C) A fluorescence photomicrograph and (D) corresponding hematoxylin and eosin stain after Liporubicin-based ILP reveal weak and sporadic signaling confined to the tumor periphery and surrounding lung parenchyma (original magnification × 40).

COMPARISON OF FREE AND LIPOSOMAL DOXORUBICIN. Isolated lung perfusion with free doxorubicin resulted for both drug doses in a significantly higher drug uptake in tumors and lung tissue than Liporubicin ($p < 0.01$). However, the tumor/normal tissue drug ratio was higher for Liporubicin than for free doxorubicin at a drug dose of 100 μg (0.53 ± 0.5 vs 0.27 ± 0.1; NS) and similar for both doxorubicin formulations at a drug dose of 400 μg (0.54 ± 0.2 vs 0.67 ± 0.2; NS). A higher dose of Liporubicin did therefore not result in an increase of the tumor/normal tissue drug ratio in contrast to what has been observed with free doxorubicin.

At a drug dose of 100 μg, Liporubicin resulted in a more heterogeneous drug distribution than free doxorubicin within the perfused lung (CV, 49% vs 27%; $p = 0.03$), whereas at 400 μg, the heterogenicity of drug distribution was similar for both doxorubicin formulations (CV, 28% vs 24%).

Doxorubicin Distribution in Tumor and Normal Lung Parenchyma After Isolated Lung Perfusion

Fluorescence microscopy revealed fluorescence signaling exclusively confined to the nuclei of the cells of normal lung parenchyma and tumor tissue, whatever the tissue or cell type observed. This holds true for both doxorubicin formulations and was found in all perfused lungs. Fluorescence signaling after Liporubicin-based ILP was weaker than with free doxorubicin in all perfused lungs for both drug doses applied. Both doxorubicin formulations resulted in fluorescence signaling emerging from all tissue compartments of normal lung parenchyma, including pneumocytes of the alveolar wall, endothelial cells of blood vessels, bronchial epithelium, and mesothelial cells covering the parietal pleura. In contrast, both doxorubicin formulations resulted in weak and sporadic fluorescence signaling emerging from tumors, which was mainly confined to the tumor periphery, to peritumoral inflammatory cells, and to blood vessels situated within the tumor tissue, irrespective of the drug dose applied (Fig 2 and Fig 3).

Comment

Chemotherapy for sarcoma has been extensively studied, but unfortunately, the responsiveness of these tumors to chemotherapy has been disappointingly low. Doxorubicin and ifosfamide remain the two only drugs that have consistently shown response rates ranging between 10% and 25%, and doxorubicin is in many centers considered the standard treatment for advanced soft tissue sarcoma.

Because advanced soft tissue sarcoma often presents as lung metastases without extra thoracic tumor manifestation, doxorubicin-based ILP has been designed with the aim to increase drug uptake to the lungs while sparing the systemic circulation. Isolated lung perfusion has been explored in clinical and experimental settings and has

demonstrated excellent separation between systemic and pulmonary circulations and effectively delivered high doses of the cytostatic agent to the perfused lung. The tumor response was modest in those clinical trials where no metastasectomy was performed, however, and several studies have demonstrated a lower drug uptake in the tumors than in normal lung parenchyma after ILP performed under clinical and experimental circumstances [1,2,3,27]. Effective shielding of the tumor tissues from the delivered drug [26] or uneven drug distribution within the perfused lung [16,27] may be responsible for the disappointing results after ILP.

This study was designed to assess whether ILP with liposomal-encapsulated doxorubicin resulted in a more homogeneous drug distribution in the perfused lung and a higher drug uptake in tumors compared with free doxorubicin. Liposomal delivery systems for anthracyclines have shown several advantages compared with the administration of the free drug. They have been investigated for their ability to improve drug delivery to tumors while decreasing toxicity to normal tissues [17–20].

The tumor-targeting mechanism relies on the size of liposomes, which render them difficult to extravasate through the capillaries of normal tissues outside of the reticuloendothelial system. This suggests little exposure of normal tissues to the toxicity associated with doxorubicin, in particular, a reduction of cardiac toxicity [17, 18]. In contrast, tumor angiogenesis results in discontinuous capillaries with gaps between capillary endothelial cells, which may facilitate extravasation, and accumulation of liposomes in tumor tissues [17, 18].

The rapid sequestration of liposomes by the reticuloendothelial system may be avoided by pegylation [19, 20]. Liporubicin is a recently developed pegylated liposomal doxorubicin. Passive targeting of pegylated liposomes to solid tumors has been shown under clinical conditions for various malignancies [19, 20]. We hypothesized that ILP with Liporubicin would render the treatment more tumor selective compared with free doxorubicin.

We compared the distribution of free and equimolar-dosed liposomal doxorubicin (Liporubicin) after antegrade ILP of rodent lungs containing a sarcomatous tumor. Doxorubicin-based IPL has been repeatedly explored in this experimental setting [9–12, 15, 27]. In addition to the clinical relevance of doxorubicin for the treatment of advanced sarcoma, the auto fluorescence properties of this agent allow its localization within tissues by use of fluorescence microscopy [27]. Two currently used drug dosages [11] were assessed for both doxorubicin formulations using the same experimental setting as described in a previous study [27].

The tumors were generated by subpleural injection of the tumor cell suspension, and histologic assessment including immunostaining by von Willebrand factor revealed a well-circumscribed sarcomatous tumor without spontaneous necrosis and a rich vascular network consisting of fine branching capillaries. Von Willebrand factor is a highly specific but poorly sensitive marker for endothelial cells [28]. Hence, the visible vascular network after von Willebrand immunostaining witnesses the presence of a well-developed and probably underestimated tumor vascularization.

Doxorubicin tissue concentration measurements revealed a heterogeneous spatial drug distribution in the perfused lung and a wide interanimal variability of drug tissue concentrations within the tumor and normal lung parenchyma for both doxorubicin formulations and drug dosages assessed. Both drug formulations resulted in lower drug concentrations in the tumors than in lung parenchyma for both drug dosages. Liporubicin-based ILP did obviously not result in a better tumor drug uptake compared with free doxorubicin in this model, for both drug dosages applied.

Isolated lung perfusion with free doxorubicin consistently resulted in significantly higher drug concentrations in tumors but also in normal lung tissues compared with equimolar-dosed Liporubicin for both drug dosages applied. However, a high doxorubicin concentration in normal lung parenchyma theoretically also bears the risk of increased doxorubicin-induced lung toxicity. In contrast, ILP with Liporubicin resulted in a higher tumor/normal tissue drug ratio than with free doxorubicin, but only at the lower drug dose assessed (100 μg). For the higher drug dose (400 μg), the tumor/normal lung tissue drug ratio was similar for both drug formulations. A higher dose of Liporubicin did therefore not result in a better tumor/normal tissue drug ratio in contrast to what has been observed with free doxorubicin. We speculate that the exposure of the tumors to a higher dose of free doxorubicin during ILP may overcome the cellular drug expel mechanisms in this model.

Isolated lung perfusion with a higher dose of Liporubicin led to a more homogeneous drug distribution within the perfused lung compared to the lower drug dose. The variability in drug distribution in lung tissue was similar for both doxorubicin formulations at a dose of 400 μg but was associated with a much lower lung tissue concentration for Liporubicin than free doxorubicin. This may be an advantage with respect to clinical application because of doxorubicin-based lung toxicity [1–3, 13, 14].

Fluorescence microscopic assessment revealed for both doxorubicin formulations at both drug dosages a fluorescence signaling emerging from all tissue compartments of the perfused lungs. The signaling emerging from the tumors was only weak and sporadic, however, and was mainly confined to the tumor periphery and to the vessels situated within the tumors for both drug formulations and irrespective of the drug dose applied. The fluorescence microscopic findings parallel the drug tissue concentration measurements with a consistently lower drug uptake in tumors than in normal lung parenchyma for both doxorubicin formulations and drug doses applied.

Our results suggest that doxorubicin-based ILP in this setting results in insufficient drug accumulation in the tumors, for both doxorubicin formulations and both drug doses applied. We speculate that the poor tumor drug uptake observed for both doxorubicin formulations may

be related to tumor-inherent mechanisms [26]. Our results do not suggest that these results are related to a lack of tumor vascularization, because histologic and immunohistochemical staining revealed a dense vascular network present throughout the tumor and the absence of spontaneous tumor necrosis.

A criticism of our experimental setting might address the relatively short exposure time of the perfused lung to the cytostatic agents. Nevertheless, the exposure time was sufficient to enable a significant drug uptake in the perfused lung in all animals assessed. Moreover, another in vivo study that assessed doxorubicin distribution in murine tumors by use of fluorescence microscopy after intravenous injection also demonstrated a lower drug uptake in tumors than in surrounding normal tissues [29].

Another criticism may involve the route of sarcoma cell injection to the rodent lung in our model. Metastases in humans are presumed to be generated by blood-borne tumor cell seeding. In our model, the tumor was generated by direct subpleural injection of the tumor cell suspension, which may not reflect the clinical situation. In a previous study, we compared the generation of MCA-induced sarcoma in rodent lungs either by the intravenous or the subpleural route of tumor cell injection [21]. Both techniques resulted in tumors of similar histologic features and vascularization. The subpleural injection resulted in a more reliable and reproducible tumor growth of one single tumor but with a bronchial artery–derived vasculature. Nevertheless, the comparison of antegrade and retrograde doxorubicin-based ILP in this model revealed a similar tumor drug uptake for both techniques, although one might theoretically expect a better tumor drug uptake after retrograde ILP, given the bronchial artery–derived tumor vascularization [27]. In addition, the tumor drug uptake after ILP in our setting was comparable to that reported in other studies where the tumors were generated by intravenous cell injection.

In conclusion, ILP with free doxorubicin resulted in a higher drug uptake in tumors and lung parenchyma than equimolar dosed liposomal doxorubicin for both drug doses applied. The tumor/normal tissue drug ratio was higher for liposomal doxorubicin at a lower drug dose and similar for both formulations at a higher drug dose. Both doxorubicin formulations resulted in a heterogeneous drug distribution in the perfused lung and in consistently lower drug uptake in tumor compared with lung parenchyma, irrespective of the drug dose applied. Future investigations must focus on means for enhanced and selective tumor uptake of cytostatic agents during isolated lung perfusion.

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References

1. Minchin RF, Johnston MR, Aiken MA, Boyd MR. Pharmacokinetics of doxorubicin in isolated lung of dogs and humans perfused in vivo. J Pharmacol Exp Ther 1984;229:193–8.
2. Johnston MR, Minchen RF, Dawson CA. Lung perfusion with chemotherapy in patients with unresectable metastatic sarcoma to the lung or diffuse bronchioloalveolar carcinoma. J Thorac Cardiovasc Surg 1995;110:368–73.
3. Burt ME, Liu D, Abolhoda A, et al. Isolated lung perfusion for patients with unresectable metastases from sarcoma: a phase I trial. Ann Thorac Surg 2000;69:1542–9.
4. Ratto GB, Toma S, Civalleri D, et al. Isolated lung perfusion with platinum in the treatment of pulmonary metastases from soft tissue sarcomas. J Thorac Cardiovasc Surg 1996; 112:614–22.
5. Pass HI, Mew DJ, Lranda KC, Temeck BK, Donington JS, Rosenberg SA. Isolated lung perfusion with tumor necrosis factor for pulmonary metastases. Ann Thorac Surg 1996;61: 1609–17.
6. Putnam JB. New and evolving treatment methods for pulmonary metastases. Semin Thorac Cardiovasc Surg 2002;14: 49–56.
7. Schroder C, Fisher S, Pieck AC, et al. Technique and results of hyperthermic (41°C) isolated lung perfusion with high-doses of cisplatin for the treatment of surgically relapsing or unresecable lung sarcoma metastases. Eur J Cardiothorac Surg 2002;22:41–6.
8. Hendriks JM, Grootenboers MJ, Schramel FM, et al. Isolated lung perfusion with melphalan for resectable lung metastases: a phase I clinical trial. Ann Thorac Surg 2004;78:1919–27.
9. Weksler B, Schneider A, Ng B, Burt ME. Isolated single lung perfusion in the rat: an experimental model. J Appl Physiol 1993;74:2736–9.
10. Weksler B, Ng B, Lenert JT, Burt ME. Isolated single lung perfusion with doxorubicin is pharmacokinetically superior to intravenous injection. Ann Thorac Surg 1993;56:209–14.
11. Weksler B, Lenert J, Ng B, Burt M. Isolated lung perfusion with doxorubicin is effective in eradicating soft tissue sarcoma lung metastases in a rat model. J Thorac Cardiovasc Surg 1994;107:50–4.
12. Abolhoda A, Brooks A, Nawata S, Kaneda Y, Cheng H, Burt M. Isolated lung perfusion with doxorubicin prolongs survival in a rodent model of pulmonary metastases. Ann Thorac Surg 1997; 64:181–4.
13. Furrer M, Lardinois D, Thormann W, et al. Isolated lung lung perfusion: single-pass system versus recirculating blood perfusion in pigs. Ann Thorac Surg 1998;65:1420–5.
14. Furrer M, Lardinois D, Thormann W, et al. Cytostatic lung perfusion by use of an endovascular blood occlusion technique. Ann Thorac Surg 1998;65:1523–8.
15. Weksler B, Burt M. Isolated lung perfusion with antineoplastic agents for pulmonary metastases. Chest Surg Clin N Am 1998;8:157–82.
16. Krueger T, Kuemmerle A, Kosinski M, et al. Cytostatic lung perfusion results in heterogeneous spatial regional blood flow and drug distribution. Evaluation of different cytostatic lung perfusion techniques in a porcine model. J Thorac Cardiovasc Surg 2006;132:304–11.
17. Papahadjopoulos D, Allen TM, Gabizon A, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci U S A 1991;88:11460–4.
18. Gabizon AA. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res 1992;52:891–6.
19. Lyass O, Uziely B, Ben-Yosef R, et al. Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma. Cancer 2000;89: 1037–47.
20. Harrington KJ, Mohammadtaghi S, Uster PS, et al. Effective targeting of solid tumors in patients with locally advanced

cancers by radiolabeled pegylated liposomes. Clin Cancer Res 2001;7:243–54.
21. Pan Y, Krueger T, Tran N, Yan H, Ris HB, McKee TA. Evaluation of tumor vascularisation in two rat sarcoma models for studying isolated lung perfusion. Injection route determines the origin of tumor vessels. Eur Surg Res 2005;37:92–9.
22. Kuemmerle A, Krueger T, Dusmet M, et al. A validated assay for measuring doxorubicin in biological fluids in an isolated lung perfusion model: matrix effect and heparin interference strongly influence doxorubicin measurements. J Pharm Biomed Anal 2003;33:475–94.
23. Chin DL, Lum BL, Sikic BI. Rapid determination of pegylated liposomal doxorubicin and its major metabolite in human plasma by ultraviolet visible high performance liquid chromatography, J Chromatogr B Analyt Technol Biomed Life Sci 2002;779:259–69.
24. Andrejevic-Blant S, Hadjur C, Ballini JP, et al. Photodynamic therapy of early squamous cell carcinoma with tetra (m-hydroxyphenyl) chlorin: optimal drug-light interval. Br J Cancer 1997;76:1021–8.
25. Rasband WS. ImageJ. Betheseda, MD: US National Institutes of Health; 1997–2007. Available at: http://rsb.info.nih.gov/ij/.
26. Jain RK. Transport of molecules across tumor vasculature. Cancer Metastasis Rev 1987;6:559–93.
27. Krueger T, Kuemmerle A, Andrejevic-Blant S, et al. Antegrade versus retrograde isolated lung perfusion: Doxorubicin uptake and distribution in a sarcoma model. Ann Thorac Surg 2006;82:2024–30.
28. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. J Histochem Cytochem 2006;54:385–95.
29. Primeau AJ, Rendon A, Hedley D, Lilge L, Tannock IF. The distribution of the anticancer drug doxorubicin in relation to blood vessels in solid tumors. Clin Cancer Res 2005;11:8782–8.

Requirements for Maintenance of Certification in 2008

Diplomates of the American Board of Thoracic Surgery (ABTS) who plan to participate in the Maintenance of Certification (MOC) process which will begin in 2008 must hold an unrestricted medical license in the locale of their practice and privileges in a hospital accredited by the JCAHO (or other organization recognized by the ABTS). In addition, a valid ABTS certificate is an absolute requirement for entrance into the Maintenance of Certification process. If your certificate has expired, the only pathway for renewal of a certificate is to take and pass the Part I (written) and the Part II (oral) certifying examinations.

The names of individuals who have not maintained their certificate will no longer be published in the American Board of Medical Specialties directories. Diplomates' names will be published upon successful completion of the Maintenance of Certification process.

The CME requirements are 30 Category I credits earned during each year prior to application. At least half of these CME hours need to be in the broad area of thoracic surgery. Category II credits are not allowed. Interested individuals should refer to the Booklet of Information for Maintenance of Certification for a complete description of acceptable CME credits.

Diplomates in the Maintenance of Certification process will be required to complete all sections of the SESATS self-assessment examination. It is not necessary for Diplomates to purchase SESATS individually because it will be sent to them after their application has been approved.

Diplomates may apply for Maintenance of Certification in the year their certificate expires, or if they wish to do so, they may apply up to two years before it expires. However, the new certificate will be dated 10 years from the date of expiration of their original certificate or most recent recertification certificate. In other words, going through the Maintenance of Certification process early does not alter the 10-year validation. Diplomates certified prior to 1976 (the year that time-limited certificates were initiated) are also required to participate in MOC if they wish to maintain valid certificates.

The deadline for submission of application for the Maintenance of Certification is May 10 of each year. All ABTS diplomates will receive a letter from the Board outlining their individual timeline and MOC requirements. A brochure outlining the rules and requirements for Maintenance of Certification in thoracic surgery is available upon request from the American Board of Thoracic Surgery, 633 North St. Clair St, Suite 2320, Chicago, IL 60611; telephone (312) 202-5900; fax (312) 202-5960; e-mail: info@abts.org. This booklet is also published on the website: www.abts.org.