Doxorubicin Gradients in Human Breast Cancer

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ABSTRACT

Ten patients with locally advanced breast cancer were given doxorubicin i.v., and an incision biopsy was subsequently taken. Doxorubicin autofluorescence was examined using computerized laser scanning microscopy, and microvessels were immunostained in the same sections. Overlays of both pictures revealed doxorubicin gradients in tumor islets with high concentrations in the periphery and low concentrations in the center of the tumor islets. Gradients were most pronounced shortly after the injection, but they could still be detected 24 h later. No gradients were observed in connective tissue. This study demonstrates a serious risk of the drug not reaching all of the cancer cells in those cases in which the cancer cells are densely packed in islets. The efficacy of drug treatment will thus depend on the histology of the tumor tissue.

INTRODUCTION

Epithelial cancer is currently the most common cause of cancer-related death. Although the normal architecture of intact epithelium is lost, in epithelial cancer, the frequent finding of desmosomes and occasionally tight junctions (1) indicate that the malignant epithelium may at least partially retain its function as a barrier, e.g., against drugs. A low density of blood capillaries may limit tumor growth (2) because of reduced delivery of oxygen and nutrients. However, during chemotherapy, reduced delivery of anticancer drugs may mean a growth advantage for the most remote tumor cells. Drug transport out of the blood capillaries and into the tissue can occur through the cells (transcellular pathway) and through the interstitium (paracellular pathway; Ref. 3). Doxorubicin is one of the most effective agents for the treatment of epithelial cancer. However, the drug rarely eliminates all cancer cells in patients with advanced disease, including breast cancer. Previously, in vitro experiments using multicell spheroids have demonstrated doxorubicin gradients (4, 5). This study was undertaken to investigate whether such gradients can be found in vivo as well.

At the cellular level, P-glycoprotein (6–8) and multidrug resistance protein (9, 10) can mediate drug resistance by decreasing transport to the target. Drug pumping by these proteins across the plasma membrane resulted in lower intracellular drug concentrations (11–14). At the tissue level, drug transport barriers have been recognized for large molecules (15, 16), which, because of their low diffusion rate, depend largely on convective flows for their transport. Furthermore, they may also be hampered by the high hydrostatic pressure outside the microvessels, conditions that often occur in solid tumors (15, 16). The net transport of small molecules (such as most drugs with a $M_r < 1000$) by diffusion may slow down as a result of binding to relatively large molecules that move at a much slower speed (17). Moreover, when the transport rate across the plasma membrane is low in combination with strong intracellular binding, drug transport through the cells can be slowed down even more drastically. Then, just after i.v. injection, after a fast rise in the blood concentration of the drug, the intracellular free drug concentration will rise slowly during influx at the front (at the side of the blood vessel). Because drug transport through the plasma membrane at the back of the cell (toward the neighboring cell) depends on this intracellular free drug concentration, it will also be slow and rise slowly (the latter is due to strong intracellular binding). In combination with a low paracellular transport in epithelial cancer, the result may be a nonhomogenous drug distribution in tissue. It must be noted that under these conditions, the drug molecules are not hampered by slow convective flows in the opposite direction driven by a pressure gradient.

In our search for gradients in vivo, we have used computerized fluorescence microscopy, exploiting the autofluorescence of doxorubicin. Earlier reports on doxorubicin tissue distribution in paraffin sections used either direct autofluorescence measurement by conventional fluorescence microscopy (18) or indirect measurement using antibodies against the drug (19). The drug may be partly lost during sample preparation with both methods. Both studies reported heterogeneous drug distribution throughout the tissue. To avoid such drug loss, we used cryosections for measuring doxorubicin fluorescence without further contact of the sections with solvents. In mice, Hoechst 33342 dye has been reported to show steep gradients in biopsies alongside microvessels (20). Previously, doxorubicin gradients in tissue have been observed in mice, using an ovarian tumor model and i.p. injection (21). In our search for doxorubicin gradients after i.v. injection of the drug in patients, we overlayed digital pictures of doxorubicin fluorescence with immunohistochemistry pictures of CD31 (staining of endothelial cells). This procedure allowed us to determine the spatial distribution of doxorubicin in relation to microvessels.
Patients with locally advanced breast cancer were treated according to a moderately high-dose chemotherapy protocol in which doxorubicin was an important ingredient (22, 23).

**MATERIALS AND METHODS**

**Chemotherapy.** Chemotherapy consisted of moderately high-dose doxorubicin (90 mg/m² body surface) and high-dose cyclophosphamide (1000 mg/m²) on day 1, followed by granulocyte macrophage colony-stimulating factor (250 µg/m²) s.c. or i.v. on days 2–11. Cycles (total, six cycles) were repeated every 3 weeks. Tumor biopsies of approximately 0.5 × 0.5 × 0.5 cm in size were taken under local or general anesthesia after oral consent and snap-frozen in liquid nitrogen. Cryosections of the whole biopsies were cut for imaging of doxorubicin fluorescence and subsequent immunostaining.

**Doxorubicin Imaging by CLSM.** A CLSM (type TCS 4D; Leica Heidelberg, Heidelberg, Germany) was used. Fluorescence images were made using the autofluorescence of doxorubicin. A krypton-argon laser line (488 nm) was used for excitation of doxorubicin, and a long pass filter (550 nm) was used for detection of the emitted light. Images at low and high resolution were obtained throughout the biopsy. We did not observe any change in the doxorubicin distribution during handling of the samples, even after keeping the sample at room temperature for 5 h. Integrated nuclear doxorubicin fluorescence was obtained using Leica Q500MC Quantimet software (version V01.01; Leica Cambridge Ltd., Cambridge, United Kingdom).

**Immunohistochemical Staining.** The endothelial cells of blood vessels were stained immunohistochemically, using the same frozen sections as for doxorubicin imaging, as follows. The cryosections were fixed in acetone on ice or in 4% paraformaldehyde followed by acetone on ice. They were incubated for 1 h with mouse anti-human CD31 monoclonal antibody (clone JC/70A; DAKO A/S, Glostrup, Denmark), followed by a 30-min incubation with rabbit antinmouse biotin complex (DAKO). Peroxidase was blocked with 3% hydrogen peroxide for 30 min before incubation with ABCComplex/horseradish peroxidase (DAKO) for 30 min. A red color was developed in 10–30 min with the 3-amino-9-ethylcarbazole kit (Vector Laboratories, Inc., Burlingame, CA), and nuclei were counterstained with hematoxylin. Hematoxylin images were obtained by combining red, green, and blue transmission images using the same optics. Images at low and high resolution were again obtained throughout the biopsy with CLSM. Overlaying of pictures (doxorubicin and immunostaining) was accomplished using Photo-Paint software (version 6.00; 1995; Corel Co., Ottawa, Canada).

**Materials.** Doxorubicin HCl was obtained from Montedison Nederland (Rotterdam, the Netherlands). DNase I was purchased from Boehringer Mannheim (Mannheim, Germany).

**RESULTS**

**Doxorubicin Distribution in Breast Cancer.** In the cryosections, doxorubicin was seen as a nuclear fluorescence clearly distinguishable from background fluorescence (Fig. 1), which was predominantly from the cytoplasm (Fig. 1A). For each patient (Fig. 1, B–I), both doxorubicin distribution patterns and CD31 immunohistochemical staining of the same area of the same section are represented. Doxorubicin gradients were observed most clearly in the ductal invasive carcinomas showing tumor islets in the biopsies, which were taken at 2 h after injection (Fig. 1, D–F). These doxorubicin gradients were not detected in the connective tissue. Also, no clear gradients were observed in patients with invasive lobular cancer with more connective tissue and strands of tumor cells (Indian files; Fig. 1, B and C). Occasionally, connective tissue showed bands of...

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2 The abbreviation used is: CLSM, confocal laser scanning microscope/ microscopy.
fluorescence (Fig. 1, D, F, and G); however, this fluorescence could be distinguished morphologically from typical nuclear doxorubicin fluorescence.

Gradient Steepness. In four patients, the average nuclear doxorubicin fluorescence was plotted against the distance from the nearby rim of the islet to demonstrate the steepness of the gradient (Fig. 2). Less steep gradients were found 24 h after injection, as compared to gradients at 2 h after injection.

Distribution at Lower Magnification. Fig. 3 shows an overview over a larger part of one of the sections in pseudocolors (red, doxorubicin; green, CD31) with the highest doxorubicin concentrations around the microvessels (10× magnification) and gradients extending from the capillaries (40× magnification).

Previously, doxorubicin concentrations (averaged per weight of tissue) in biopsies have been measured by high-performance liquid chromatography (24, 25). No metabolites could be detected in breast cancer biopsies (25), indicating that the nuclear fluorescence comes only from doxorubicin itself. CD31 immunohistochemical staining indicated the presence of capillary vessels in the connective tissue. To compare the data from tumor with structurally similar normal tissue, skin biopsies were taken from three patients after doxorubicin administration. Doxorubicin gradients were found in all three of the biopsies examined (data not shown).

DISCUSSION

Generally, small molecules with $M_r < 1000$ show a more rapid diffusion into tissues than large molecules, such as antibodies or viral vectors. Under conditions of extensive intracellular binding and a low plasma membrane transport rate, penetration in the tumor islets of such drugs, such as doxorubicin ($M_r 543.54$), can be slowed down considerably. Here we show the slow penetration of the small-molecule drug doxorubicin in patients, resulting in gradients in clinical biopsies of solid tumors. Cells in the center of the tumor islets, which are the most remote from the microvessels, are exposed to lower drug concentrations in the surrounding extracellular fluid compared to the cells in the periphery of the tumor islets. In general, the three-dimensional gradient toward the center of an islet may be steeper than the observed two-dimensional image, due to the fact that the cross-sections of the islets generally will not run through the center of the islets. In that case, the distance from a cell inside such an islet to the islet boundary will be shorter than that observed in the two-dimensional cross-sections; in reality, the doxorubicin concentration gradient will be even steeper than that measured. It could be argued that when the gradient reverses during drug clearance from the blood, the drug concentration in cells at the periphery might become lower when compared to cells at the center of the islet. However, back supply from the inner layers will buffer this fall in concentration. Diffusion will be slow through both the transcellular and the paracellular routes: it will be slow through the paracellular route because of the small volume of the intercellular space; and it will be slow through transcellular route because of slow membrane passage and high intracellular binding (mainly to DNA). To understand the generation of doxorubicin gradients, further development of transport models determining the relative contributions of factors affecting the total transport will be necessary.

As an alternative scheme for doxorubicin administration per bolus injection, a continuous 96-h infusion has been applied to patients for the treatment of metastatic breast cancer and sarcoma. This method of administration had a favorable effect on life-threatening heart toxicity (26, 27). The comparable clinical antitumor efficacy at the relatively low plasma concentrations during infusion, when compared to the high concentrations during the first few hours after i.v. injection, could partly be due to a more homogenous doxorubicin tissue distribution in cancer islets. The kinetics of drug in the blood shows that after i.v. injection, the doxorubicin plasma concentration declined from...
15 to 3 μg/liter in the 6–96-h period after injection. During the first few hours after injection, the concentration declines much more rapidly from a level 2 orders of magnitude higher (24). With low effective drug diffusion into the tumor islet, this profile leads to the development of a gradient, especially in the first few hours, when the outer tumor cells of an islet are exposed to relatively high concentrations. Later, e.g., at 24 h, the tissue gradient is less steep due to diffusion and the decline of the plasma concentration. During continuous infusion at a comparable total dose, the plasma concentration gradually rose to 15 μg/liter at 24 h after the start of the infusion (28). In that case, the high initial concentrations after i.v. injection are missing; therefore, the tumor islets will experience a much more steady concentration resulting in gradients that are less steep.

Doxorubicin-based chemotherapy of breast cancer followed by surgery (22, 23) can be an effective treatment for locally advanced disease. To avoid possible memory effects of low concentration resulting in gradients that are less steep due to diffusion and the decline of the plasma concentration. During continuous infusion at a comparable total dose, the plasma concentration gradually rose to 15 μg/liter at 24 h after the start of the infusion (28). In that case, the high initial concentrations after i.v. injection are missing; therefore, the tumor islets will experience a much more steady concentration resulting in gradients that are less steep.

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