The Distribution of the Anticancer Drug Doxorubicin in Relation to Blood Vessels in Solid Tumors

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Abstract

Purpose: Anticancer drugs gain access to solid tumors via the circulatory system and must penetrate the tissue to kill cancer cells. Here, we study the distribution of doxorubicin in relation to blood vessels and regions of hypoxia in solid tumors of mice.

Experimental Design: The distribution of doxorubicin was quantified by immunofluorescence in relation to blood vessels (recognized by CD31) of murine 16C and EMT6 tumors and human prostate cancer PC-3 xenografts. Hypoxic regions were identified by injection of EF5.

Results: The concentration of doxorubicin decreases exponentially with distance from tumor blood vessels, decreasing to half its perivascular concentration at a distance of about 40 to 50 μm. The mean distance from blood vessels to regions of hypoxia is 90 to 140 μm in these tumors. Many viable tumor cells are not exposed to detectable concentrations of drug following a single injection.

Conclusions: Limited distribution of doxorubicin in solid tumors is an important and neglected cause of clinical resistance that is amenable to modification. The technique described here can be adapted to studying the distribution of other drugs within solid tumors and the effect of strategies to modify their distribution.

The majority of research on drug resistance in tumors has emphasized genetically mediated mechanisms expressed at the cellular level that lead to intrinsic resistance of the individual tumor cells to available anticancer drugs. However, the in vivo response of solid tumors to chemotherapy is not just a function of the sensitivities of the constituent cells. Solid tumors are complex societies where cells reside within variable micro-environments that can influence their response to chemotherapy. Important factors that influence the response of tumors to chemotherapy include variable vascular density and blood flow, regions of acute and chronic hypoxia that can influence drug uptake and activity, and the requirement for drugs to penetrate through solid tissue. Anticancer drugs gain access to solid tumors via the circulatory system, and must penetrate the extravascular space to reach all cancer cells at a sufficient concentration to cause lethal toxicity. Relatively little research has been done to investigate the distribution of drugs in relation to blood vessels.

Initial evidence for limited penetration through tissue of the commonly used anticancer drug doxorubicin was obtained by study of the distribution of its fluorescence in multicellular spheroids (1). Subsequently, our group and others have used a multicellular layer model to study the penetration of anticancer drugs through tissue (2–5). This model provides evidence that the tissue is a barrier to the distribution of anticancer agents, and that the penetration of doxorubicin is particularly poor.

The implantation of tumors into transparent chambers of the skin of rats permits microscopic evaluation of tumor microcirculation in vivo and has provided additional evidence of limited penetration through tissue (6–8). Lankelma et al. (9) obtained biopsies of human breast cancer after patients were treated with doxorubicin and by exploiting the autofluorescent properties of the drug, they provided a semiquantitative assessment of doxorubicin gradients further supporting the hypothesis that poor penetration may limit chemotherapy.

Here, we quantify and model the penetration/gradient of doxorubicin relative to multiple blood vessels in two murine tumors and a human cancer xenograft in mice and incorporate the hypoxia marker EF5 to identify regions of hypoxia. We use wide field computerized microscopy to show and quantify the gradients of doxorubicin in relation to several hundred vessels within a given tissue section. We show that many tumor cells are not exposed to the drug following a single i.v. injection and provide a model to study agents that might modify drug distribution.

Materials and Methods

Drugs and reagents. Doxorubicin (Pharmacia, Mississauga, Ontario, Canada) was provided by the hospital pharmacy as a solution at a concentration of 2 mg/mL. The hypoxia-selective agent EF5 and Cy5-conjugated anti-EF5 antibody were provided by Dr. C. Koch, Philadelphia, PA. Purified rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1) monoclonal antibody was purchased from...
BD PharMingen (Mississauga, Ontario, Canada) and the Cy3-conjugated goat anti-rat IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Tumor models.** Experiments were done using the mouse mammary sarcoma EMT6, the human prostatic carcinoma xenograft PC-3, and the mouse mammary adenocarcinoma 16C. The EMT6 model was selected as a well-characterized murine tumor that grows in *vivo* and *in vitro*, for which we have studied drug penetration in multicellular layers (3, 4). The PC-3 tumor was selected as a human xenograft that grows readily in nude mice (10). The 16C tumor was selected as one of the very few murine tumors that respond to doxorubicin (11).

EMT6 and PC-3 cells were maintained as monolayers in α-MEM supplemented with 10% FCS at 37°C in a humidified atmosphere of 95% air plus 5% CO₂. Tumors were generated by the injection of 10⁶ exponentially growing cells into the right flank of 6- to 8-week-old BALB/c (EMT6) or athymic nude male mice (PC-3). Cells from 16C tumors do not grow in culture: tumors were generated by passage in C3H mice following implantation from frozen stock. Mice were maintained in the animal colony at the Ontario Cancer Institute, University of Toronto (Toronto, Canada). Mice were housed five per cage. Sterile tap water and food were given ad libitum. All procedures were carried out following approval of the Institutional Animal Care Committee.

**Tumor growth and response to doxorubicin.** Tumor-bearing mice were divided randomly into groups of 9 to 10, and treatment with doxorubicin was initiated when the diameter of tumors was 8 to 10 mm. One group was selected randomly as the control, and in the other, mice received 8 mg/kg of doxorubicin as a single i.p. injection. Control mice were given equal volumes of PBS. The longest perpendicular diameters of tumors were measured twice weekly to document tumor growth. Tumor measurements were converted to tumor volume (V) using the formula: $V = W^2 \times Y/2$; where W and Y are the smaller and larger perpendicular diameters, respectively. The body weight of mice was also measured twice weekly.

**Distribution of doxorubicin in tumors.** Tumor-bearing mice were divided randomly into groups of six and were treated when the mean tumor diameter was in the range 8 to 12 mm. Animals received 0.2 mL of 10 mmol/l EF5 i.p. 3 hours prior to chemotherapy. In these experiments, doxorubicin was injected i.v. In most experiments, doxorubicin was given at a dose of 30 mg/kg to facilitate detection and quantification of autofluorescence; this dose would have ultimately led to the death of the animals, and in some experiments, a tolerated dose of 6 mg/kg was injected. Animals were killed and tumors excised at 5, 10, 20, 40, and 180 minutes following injection of doxorubicin. Tumors were embedded immediately in optimum cutting temperature compound, frozen in liquid nitrogen, and stored at −70°C prior to tissue sectioning and immunohistochemical staining. Single cryostat sections 10 μm thick were cut from each tumor, mounted on glass slides, and allowed to air-dry.

Doxorubicin autofluorescence was detected using a Zeiss Axiovert 200M inverted microscope with a 100 W HBO mercury light source equipped with a 530 to 560 nm excitation and a 573 to 647 nm emission filter set. Tissue sections were imaged with a FLIAR 10×/0.50 NA lens and captured with a Roper Scientific CoolSnap HQ CCD camera. Tissue sections were tiled using a motorized stage so that the distribution of doxorubicin was obtained for the entire tissue section (up to 2 × 2 mm²). All images were captured in 8-bit signal depth and subsequently pseudo-colored.

Blood vessels in tissue sections were recognized by the expression of CD31 on endothelial cells, and hypoxic regions were recognized by uptake of EF5. Subsequent to imaging of doxorubicin, tissue sections were fixed in acetone for 10 minutes. Sections were then washed in PBS and blocked with a protein-blocking reagent (ID Labs, Inc., London, Ontario, Canada) for 15 minutes to prevent nonspecific antibody binding. They were dually stained with a rat anti-CD31 (1/100) and cyanine-5-conjugated mouse anti-EF5 (1/50) antibody cocktail for 1 hour in a humidified chamber (antibody dilutions indicated in parentheses). Finally, sections were washed in PBS and stained with a Cy3-conjugated goat anti-rat IgG secondary (1/400) for 40 minutes to recognize rat anti-CD31. Sections were then washed in PBS and air-dried for fluorescent imaging. Tissue sections were re-imaged in an identical way to that used to capture doxorubicin fluorescence. Cy3 fluorescence representing endothelial cells was visualized using 530 to 560 nm excitation and 573 to 647 nm emission filter sets. Cy5 fluorescence, representing hypoxic regions, was visualized with 630 to 650 nm excitation and 665 to 695 nm emission filters. Tissue sections were subsequently stained with hematoxylin to delineate nuclear morphology and regions of necrosis.

Composite images of doxorubicin, CD31, and EF5 were generated using Media Cybernetics Image Pro PLUS (version 5.0). Images displaying anti-CD31 staining were converted to a black and white binary image, and objects ≤5 μm², based on conservative estimation of minimal capillary diameter (12), were removed. The resultant image was overlayed with the corresponding field of view displaying doxorubicin fluorescence resulting in an 8-bit black and white image with blood vessels identified by an intensity of 255 (white) and doxorubicin ranging from 0 to 254. Regions of interest were selected from each tissue section and were on average 1.6 mm² (0.4 μm²/pixel). Areas of necrosis and staining artifacts were excluded. To minimize noise from tissue autofluorescence, a minimum signal level just below threshold for the detection of doxorubicin was set for each tissue section; this was based on an average background reading from regions without nuclear fluorescence/staining. The pixel intensity and distance to the nearest vessel for all pixels within the selected region of interest above threshold were measured with a customized algorithm.

Doxorubicin intensity (I) was averaged over all pixels at a given distance (x) to the nearest vessel and plotted as a function of distance to the nearest vessel.

Although the boundary was well defined, EF5 staining is diffuse due to acute and chronic regions of hypoxia permitting only a semiquantitative analysis of hypoxia. Thus, only the distance (x) to regions of chronic hypoxia were estimated by measuring the length (μm) from the edges of selected vessels to the onset of regions of chronic hypoxia, marked by EF5 staining. Approximately 10 to 15 vessels were selected from each tissue section for quantification. Blood vessel density, defined as percentage of positive CD31 staining per unit area, was quantified using Image J software.

**Statistical analysis.** Data are presented as mean ± SE. To test for differences in mean blood vessel density, mean distance of hypoxic regions from the nearest blood vessel, and characteristic penetration length (L) as a function of time between tumors, one-way ANOVA was done (Sigma Stat, Chicago, IL). Nonlinear regressions were carried out using GraphPad Prism (San Diego, CA). To test for significant differences in characteristic penetration length (L) and perivascular fluorescence intensity of doxorubicin (I₂) between consolidated data for tumor types, F tests were done (GraphPad Prism). P < 0.05 was considered statistically significant.

**Results**

**Sensitivity of tumors to doxorubicin.** The growth of PC-3 (n = 9), 16C (n = 10), and EMT6 (n = 10) tumors following a single treatment with doxorubicin (8 mg/kg), or vehicle controls is shown in Fig. 1. Growth delay caused by doxorubicin is expressed as the number of days for tumors to undergo a 10-fold increase in volume from 100 to 1,000 mm³ as compared with controls. The 16C tumors had the best response to doxorubicin (growth delay ~13 days), EMT6 had an intermediate response (growth delay ~5 days), whereas PC-3 tumors showed complete resistance (Table 1).
Mice bearing the PC-3 and 16C tumors showed no signs of toxicity following injection of 8 mg/kg of doxorubicin, and did not lose weight; BALB/c mice bearing EMT6 tumors showed weight loss, with 4 of 10 animals dying at day 18.

**Distribution of doxorubicin.** Representative three-color composite images showing the distribution of doxorubicin (blue) in relation to blood vessels (red) and hypoxic regions (green) in tissue sections from PC-3, 16C, and EMT6 tumors are shown in Fig. 2. There was no significant difference between the tumors in the relative frequency of blood vessels in tumor sections (P = 0.8; Table 1). The mean distance from blood vessels to regions of chronic hypoxia was statistically greater in PC-3 than in EMT6 and 16C (P < 0.05), with no detectable difference between EMT6 and 16C tumors (Table 1).

Images from all three tumors show that high concentrations of doxorubicin are localized around blood vessels, whereas many regions of the tumors do not contain detectable doxorubicin. Areas of tumor sections without detectable doxorubicin include all of the hypoxic and adjacent regions, and hematoxylin staining of the same sections (data not shown) reveals apparently viable tissue within them. There are also occasional CD31-positive blood vessels without surrounding doxorubicin, some of them within regions of hypoxia; these vessels presumably did not contain circulating blood at the time of injection, leading to acute hypoxia.

When the intensity of doxorubicin was plotted against distance from the nearest blood vessel for tumors excised at 5, 10, 20, 40, and 180 minutes after injection of 30 mg/kg doxorubicin, a gradient was established within 5 minutes that changed minimally up to 3 hours later (one-way ANOVA test; P > 0.85 for all tumor types). As expected, doxorubicin was also found to be similarly distributed close to blood vessels following injection of a therapeutic dose of 8 mg/kg, but images have a lower signal to noise ratio. Because there was no difference in distributions as a function of time, data for varying intervals (up to 3 hours) between injection and tumor excision were consolidated to generate curves relating mean doxorubicin fluorescence to distance from the nearest vessel in PC-3, 16C, and EMT6 tumors (Fig. 3A). These curves show an exponential relationship between fluorescence intensity of doxorubicin and distance from a blood vessel (Fig. 3B) that decreases to background levels of fluorescence at ~100 μm from the nearest vessel in all three tumors. The resulting intensity profile was therefore fitted to the equation:

\[ I = I_o e^{-s\ln2/L} + C \]

**Table 1. Properties of the PC-3, 16C, and EMT6 tumors**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Growth delay (days)</th>
<th>Blood vessel density (CD31 staining/μm²)</th>
<th>Distance of hypoxic regions from nearest blood vessel (μm)</th>
<th>Initial doxorubicin fluorescence intensity “(I_o)” (arbitrary units)</th>
<th>Background constant “C” (arbitrary units)</th>
<th>Characteristic penetration length “L” (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>0</td>
<td>0.16 ± 0.02</td>
<td>142 ± 20</td>
<td>9.27 ± 0.07</td>
<td>4.57 ± 0.08</td>
<td>51.4 ± 0.8</td>
</tr>
<tr>
<td>16C</td>
<td>−13</td>
<td>0.14 ± 0.02</td>
<td>87 ± 10</td>
<td>10.27 ± 0.08</td>
<td>3.82 ± 0.09</td>
<td>39.3 ± 0.08</td>
</tr>
<tr>
<td>EMT6</td>
<td>−5</td>
<td>0.14 ± 0.02</td>
<td>104 ± 14</td>
<td>7.3 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>49 ± 1.6</td>
</tr>
</tbody>
</table>

NOTE: Data represented as mean ± SE.
*Units of fluorescent intensity are arbitrary but the scale is the same for all of the tumors.
† Indicates significant difference.
where $I_0$ is the initial pixel intensity relative to background, $C$ is the background constant, and $L$ is the characteristic penetration length [the distance ($x$) at which $I - C$ decreases to half $I_0$]. Values for the characteristic penetration length, at which the intensity of doxorubicin decreases to half its value adjacent to blood vessels, were in the range of 40 to 50 μm (Table 1). The consolidated data sets for each tumor were significantly different ($P < 0.0001$; $F$ test). The characteristic penetration length ($L$) for the 16C tumor was significantly shorter than for either the PC-3 or EMT6 tumors, with no difference between the PC-3 and EMT6 tumors. The perivascular fluorescence intensities ($I_0$) for each tumor type were all statistically different from each other ($P < 0.05$).

**Discussion**

Composite images of PC-3, 16C, and EMT6 tumors illustrating drug distribution relative to blood vessels and regions of chronic hypoxia (Fig. 2) reveal steep gradients for doxorubicin from perfused vessels. Hematoxylin staining of the same images show that a large fraction of apparently viable cells is not exposed to the drug following a single i.v. treatment, even when administered at high doses. Although...
the dose used in most of the experiments (30 mg/kg) would cause death of the animals, tolerated doses (8 mg/kg), also show limited drug penetration, suggesting that relatively few tumor cells will have access to sufficient concentration of drug to be killed. The distribution of doxorubicin in relation to tumor blood vessels is well fitted by a one-dimensional exponential decay function. Characteristic penetration lengths ($L$), representing the distance from the nearest vessel where the doxorubicin intensity decreases to half, were only in the range of 40 to 50 $\mu$m, with standard errors of the mean of 0.8 to 1.6 $\mu$m, for the three tumor types. This level of reproducibility for estimates of penetration length ($L$) among tumors of each type implies that this technique is sufficiently sensitive to detect subtle changes in doxorubicin gradients between tumors.

By limiting the analysis to two-dimensional images as representative of a three-dimensional tumor, there is a bias to overestimate the distance of a pixel to its nearest vessel, particularly at large distances, due to the presence of out-of-section vessels. This bias leads to overestimation of the characteristic penetration length ($L$), and implies that a three-dimensional analysis would indicate gradients in concentration of doxorubicin steeper than those shown in Fig. 3. To minimize the effect of this bias, and to reduce the influence of “noise” from nonspecific fluorescence, we fitted data only to distances of up to 100 $\mu$m from blood vessels. Data shown in Fig. 3 indicate that at this distance, the fluorescence due to doxorubicin is close to background levels of autofluorescence for each of the tumors that were studied (Table 1). The variables that we derive, $I_0$, $C$, and $L$ (Table 1) are determined based on several hundred vessels for each tumor and present an objective description of diffusion based on a two-dimensional image. $I_0$ reflects the concentration of doxorubicin in the blood and its ability to extravasate from the capillary, and $L$ reflects the degree of cellular uptake (due largely to binding to DNA within the nucleus and sequestration in acidic vesicles; ref. 13), as well as its diffusion constant through tissue.

The response of each tumor to doxorubicin depends on the intrinsic sensitivity of the tumor cells, and the amount of drug that reaches them. The characteristic penetration length ($L$) is not prognostic of tumor sensitivity (Table 1), because we observed poorer penetration in the drug-sensitive 16C tumor than in the resistant PC-3 tumor. However, the mean distance to regions of chronic hypoxia was larger for the PC-3 xenografts than for the murine tumors (Table 1), with the implication that a greater fraction of apparently viable cells was not exposed to detectable levels of doxorubicin. This observation could not be attributed to differences in tumor size or blood vessel density and could account, in part, for the lack of sensitivity to doxorubicin of PC-3 tumors. Also, due to the tissue scattering effects of light, it is difficult to correlate a specific dose of doxorubicin with the autofluorescence of the drug. However, results for all three tumors suggest the existence of apparently viable cells, beyond ~50 $\mu$m from the nearest vessel, that do not receive enough drug to cause toxicity. The region between the maximum penetration of drug and the onset of hypoxia (or beyond it because radiobiological experiments show that many tumors contain hypoxic cells that are viable) may contain a reservoir of clonogenic cells, which can repopulate the tumor between courses of chemotherapy. Because a large fraction of apparently viable cells is not exposed to drug, strategies that enhance drug penetration have considerable potential to increase cell killing and hence, tumor sensitivity.

The relatively poor penetration of doxorubicin is probably due to rapid uptake of the drug by perivascular cells. Doxorubicin binds avidly to the DNA within these cells, thus reducing the amount available for diffusion to distal cells. Our study provides a rigorous quantification of doxorubicin penetration length and extends the work of Lankelma et al. (9) to reinforce that chemotherapy with DNA-binding drugs, such as doxorubicin, is limited by poor penetration of tissue. In addition to quantifying gradients of doxorubicin, the methodology used here relates the distribution to multiple vessels (several hundred per image) and to regions of hypoxia. The identification of hypoxic tumor regions facilitates the recognition of viable cells that are not exposed to the drug after treatment.

Fig. 3. Background-subtracted fluorescence intensity of doxorubicin as a function of distance to the nearest vessel. $A$, linear scale for fluorescence; $B$, exponential scale for fluorescence. Distributions (□, 16C + PC-3; ○, EMT6) are fit to a one-dimensional exponential decay function (lines): $I = I_0 e^{-x/L} + C$, where $I$, pixel intensity; $I_0$, initial pixel intensity; $x$, distance to the nearest vessel; $L$, characteristic penetration length; and $C$, background constant.
Some human tumors respond well to doxorubicin in spite of its relatively poor penetration. Among the tumors studied here, the 16C tumor was much more sensitive than the EMT6 and PC-3 tumors despite similar distributions of doxorubicin. Possible reasons for this include: (a) differences in intrinsic cell sensitivity to doxorubicin, (b) differences in the number and distribution of clonogenic cells that retain the capacity to regenerate the tumor after treatment, and (c) better distribution of active metabolites of doxorubicin, such as its major metabolite doxorubicinol. Better distribution of doxorubicinol seems unlikely to convey drug sensitivity because the metabolite is considerably less toxic to cells than the parent drug (14). Sensitive tumors might have a smaller proportion of stem cells that are distributed preferentially around blood vessels. Although not apparent among the three tumors studied here, differences in blood flow, concentration, and organization of blood vessels could also contribute to differences in drug sensitivity. Sensitive tumors could have a larger proportion of perfused vessels and/or smaller intercapillary distances and hence fewer cells lying beyond the diffusion range of the drug. Moreover, repeated treatments with doxorubicin might lead to the killing of successive populations of cells at increasing distance from blood vessels (analogous to peeling an onion); although this will depend on the relative kinetics of cell killing and of repopulation from surviving cells (15, 16).

We have shown that it is possible to detect and model differences in the distribution of an anticancer drug within tissue sections from solid tumors. Future studies will extend the technique to investigate distribution profiles for other drugs and the degree to which they may be amenable to modification. For example, the fluorescent anticancer agent topotecan could also be detected by its fluorescence signature (17), whereas the distributions of cisplatin or alkylating agents might be evaluated by fluorescent antibodies targeting DNA-drug adducts (18). Related techniques can identify surrogate markers of drug penetration, such as apoptotic and/or cell cycle markers. For example Huxham et al. (5) showed that cells located distal to the vasculature in a human tumor xenograft had less disturbance of the cell cycle than proximal cells following treatment with gemcitabine, most likely due to limited penetration of the drug; the distal cells commenced cycling sooner than proximal cells and were largely responsible for repopulating the tumor.

An important application of the technique described in this article will be to determine whether drug penetration and distribution can be modified by strategies that could be used clinically. Tong et al. (7) showed deeper penetration of Cy5-labeled albumin into tumors following blockade of vascular endothelial growth factor signaling. Anti–vascular endothelial growth factor treatment has been shown to render vessels more functional, and hence, to decrease interstitial fluid pressure and improve diffusion within solid tumors (19). Additional strategies that might improve the distribution of doxorubicin include: (a) modification of the extracellular matrix to reduce cell packing so that more drug can penetrate to distal cell layers (20, 21), and (b) modifying the pH of acidic organelles that sequester basic drugs (13) such that more drug is available both for exerting toxic effects in the cell nucleus and for penetration of tumor tissue.

In summary, the results presented here show that the distribution of doxorubicin in solid tumors is poor. The sensitivity of tumors to doxorubicin is likely to depend as much on drug distribution (and the spatial distribution of clonogenic cells within the tumor) as it is on intrinsic drug sensitivity. This important and neglected cause of clinical resistance to chemotherapy is likely to apply to other drugs, including monoclonal antibodies and some molecular targeted agents. Moreover, drug distribution is amenable to modification. We describe here a method for quantifying the distribution of doxorubicin that could be extended to other agents and used to study modifiers of drug distribution.

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