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Distribution of doxorubicin to normal breast and tumour tissue in patients undergoing mastectomy

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Summary. Response to cytotoxic agents is assumed to be related to the concentration of drug achieved within tumour tissue. It is also often assumed that, given similar tissue concentrations of drug, normal tissues are less responsive to the same cytotoxic agents. This can partly be explained by the number of cells in normal tissues that are differentiated. These non dividing cells, in a stable testing phase of the cell cycle (G_0) are less susceptible to cytotoxic damage. Little is actually known about the relationship between tumour drug concentrations and those in the tissue of the tumour-bearing organ. In this study, we compared doxorubicin concentrations in paired samples of tumour and normal breast tissue from 17 previously untreated women undergoing mastectomy. The relative cellularities of both specimens were estimated by measuring their DNA content. There was wide variation in intra-tumoural doxorubicin concentrations (range, 220–1,590 ng/g). Normal tissue also showed marked inter-patient variation (range, 81–1,000 ng/g). For a single patient the tumour drug concentrations were significantly higher than those in normal breast tissue ($P < 0.05$), and tumour: normal tissue ratios ranged from 1.27 to 8.30. Where doxorubicin concentration was expressed in terms of the relative cellularity of the tissues, there was no significant difference between, drug concentrations in the tumour and those in normal breast tissue (tumour: normal ratios, 1.1:1.8). There was a significant correlation ($r = 0.76$, $P < 0.05$) between peak serum values and tumour concentrations of drug. No correlation was found between drug concentrations achieved and the histological grade or oestrogen receptor status of the breast cancer.

Introduction

Doxorubicin is known to be active against a range of solid tumours in humans [5, 6, 18]. It is widely used in the treatment of breast cancer and, with its analogue 4′epi-doxorubicin, is the first-line treatment in advanced disease. The overall response rate to doxorubicin among breast cancer patients is about 55% [1, 7, 19, 25]. This means that almost half of such patients are resistant to doxorubicin from the outset of treatment. The reasons for this phenomenon are not understood, but since mortality due to breast cancer is usually related to advancing metastatic disease, resistance to doxorubicin is a major clinical problem.

Some of the possible mechanisms of resistance to doxorubicin have been studied in vitro using either cell lines [4, 17, 23], or cells grown from patients' tumour biopsies [24]. One mechanism by which cells develop resistance to doxorubicin in vitro is the over-expression of the 180 kDa membrane glycoprotein thought to be involved in active efflux mechanisms on the membrane of normal and tumour cells [12]. This protein is expressed at varying levels in normal tissues throughout the body and may be one of the mechanisms by which some normal cells handle toxic products [13].

In patients, however, lack of response to treatment may well be due to inadequate drug delivery to the target tumour cell. Many factors are involved in determining this, such as individual variations in the pharmacokinetics of doxorubicin, tissue drug distribution and possible variations in tumour vascular patterns. Although the relative importance of these various factors is not understood, it is clear that the amount of drug taken up by tissue, particularly the amount concentrated within a solid tumour, is important in determining the likelihood of cell kill.

Few studies have measured tumour drug concentrations or normal tissue concentrations of doxorubicin in patients, partly due to the difficulty in obtaining samples from individuals who often have advanced or metastatic disease. Previous studies have also been complicated by the fact that patients may previously have undergone either hormone treatment or chemotherapy, which could well alter their responsiveness to doxorubicin [8, 16].

In this study we gave a small dose of doxorubicin to women with early-stage breast cancer who were undergoing primary surgery, from whom we could obtain samples of the tumour as well as distant, normal breast tissue for drug analysis. We examined the relationship between tissue drug concentrations and peak serum concentrations as well as the differentiated status of the tumour, measured by the presence of oestrogen receptors and by histological grade. We also used the autofluorescent properties of doxorubicin to examine its distribution in both tumour and normal breast tissue.

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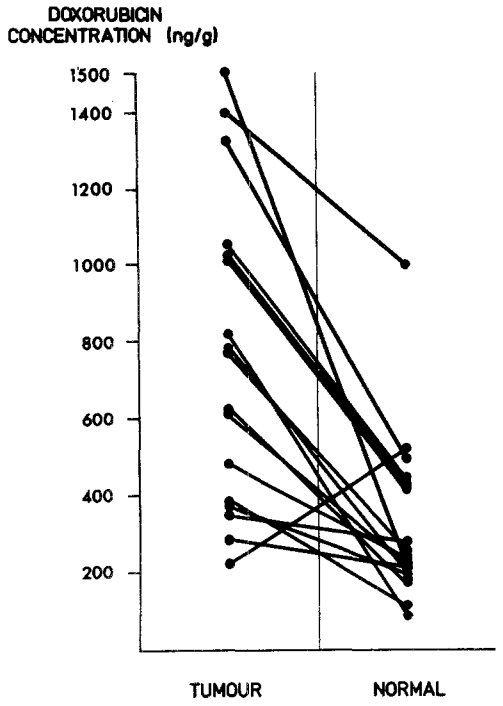

Fig. 1. Doxorubicin concentrations in breast tumour and normal tissue

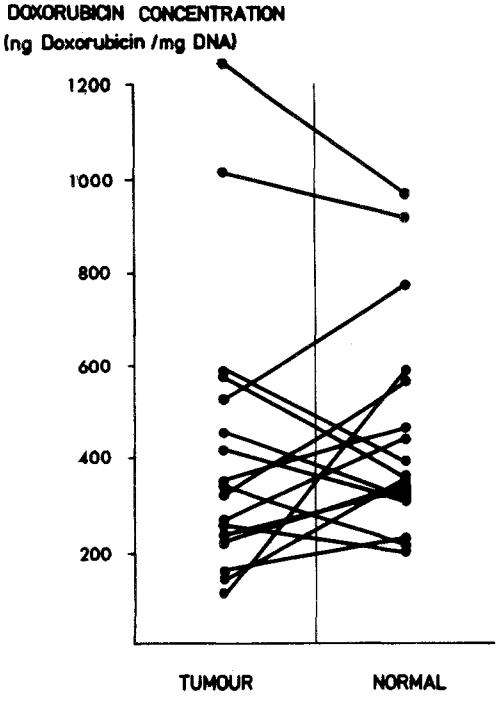

Fig. 2. Doxorubicin concentrations in breast tumour and normal breast expressed in relation to the relative cellularity of both

Materials and methods

A total of 21 women were entered into the study after informed consent was obtained. All of the patients were undergoing mastectomy for histologically proven breast cancer. Their age range was 39–70 years (mean, 52.3 years); 6 were pre-menopausal, 12 were post-menopausal and 3 were peri-menopausal. All had normal hepatic, renal and cardiac function and had undergone no previous treatment for breast cancer.

With the approval of the local ethical committee, the patients were given a small dose (25 mg i.v.) of doxorubicin (commercially available from Farmitalia, Milan, Italy). The drug dose was given approximately 1 h before the expected time of mastectomy (actual times, 58 ± 10 min). A previous study [9] has shown this time to be optimal for achieving tissue binding of doxorubicin. Venous blood was sampled at 5, 15, 30 and 60 min after drug administration; these samples were centrifuged at 200 *g* for 10 min and plasma was frozen for subsequent analysis.

On removal of the breast, histologically malignant tissue specimens were taken from the tumour and histologically benign specimens were obtained from distant, normal breast tissue. The tissue samples were immediately frozen in liquid nitrogen; part of each sample was stored for doxorubicin analysis and part was used for DNA quantification.

Doxorubicin assays. Doxorubicin and metabolite concentrations of all plasma samples were determined by HPLC analysis as previously described, with daunorubicin as an internal standard [10, 11]. Briefly, 5 ml chloroform propan-2-ol, 2:1 mixture was added to 1 ml serum and vortexed rapidly for 20 min. Three phases were separated by centrifuging at 300 *g* for 15 min, after which the aqueous upper layer was discarded. The organic layer was evaporated to dryness and redissolved in 40 µl methanol. Samples were injected onto a reverse-phase µ-Bondapak C18 column and detection was carried out by fluorescence (at 480 nm excitation, 560 nm emission).

Tumour and normal tissues were thawed, dissected free of fat and then homogenised in PBS; a 1-ml aliquot was used for the assay. Samples were pre-treated with 0.2 ml silver nitrate (33% w/v) for 10 min at 4° C to release bound drug [22], after which the extraction procedure was carried out as for plasma. As previously described, 200 ng daunorubicin was added to a sample prior to injection. The efficicency of extraction was calculated by measuring the loss of daunorubicin after extraction and expressing this as a percentage of the initial concentration. Doxorubicin concentrations were normalised by correcting for drug recovery.

DNA assay. Tissues were thawed, weighed and homogenised in buffer [20 µ*M* HEPES and 1.5 µ*M* EDTA (pH 7.4) made with 0.25 µ*M* dithiothreitol just prior to use] at a ratio of 50 mg tissue: 1 ml buffer. DNA was assayed by the modified Burton method using highly polymerised calf thymus DNA as a standard [15].

Pharmacokinetic estimations. Plasma concentration-time curves were plotted for each patient (from 0 to 60 min). Peak concentrations of drug (Co) were calculated by backward extrapolation to time 0.

Fluorescence assay. Formalin-fixed paraffin sections of tissue were prepared, mounted in Uvinert and viewed under a fluorescent microscope. Fluorescent staining was quantified by observations through a microscope with an MBV

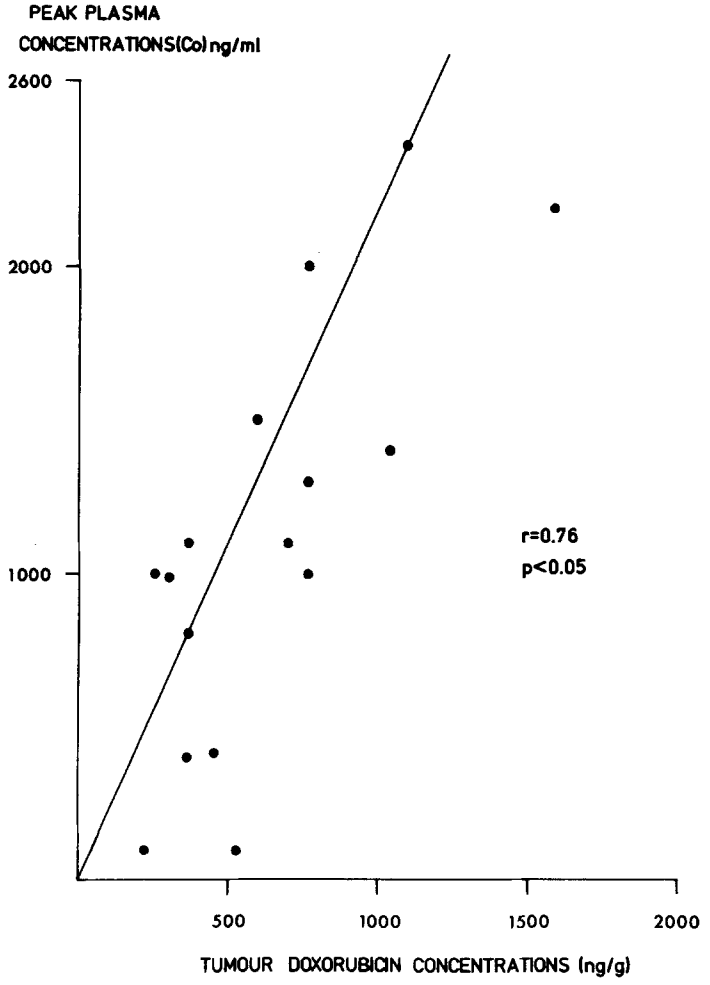

Fig. 3. Tumour doxorubicin concentrations and peak serum concentrations

compact attachment viewed at 468 nm wavelength excitation. A total of 30 separate readings were taken of four randomly chosen tumour-specimen sections. Sections of tumour and normal breast tissue from untreated patients were also viewed as controls.

Results

Of 21 patients entered into the study, 17 were evaluable; the 4 patients excluded from analysis showed no doxorubicin in either tissue or plasma specimens. It was not clear whether the latter actually indicated the absence of drug, as the extraction efficiencies in these four cases was low (<40%) and no material remained for repetition of the assay. In all other cases, extraction efficiencies ranged from 70% to 98%.

Tissue drug concentrations

The weights of tumour tissue used for analysis ranged from 140 to 810 mg. Normal tissue weights ranged from 110 to 1,340 mg. There was wide inter-patient variation in tumour concentrations of doxorubicin, ranging from 220 to 1,590 ng/g (Fig. 1). Doxorubicin concentrations in normal breast tissue also showed wide inter-individual variation, ranging from 81.7 to 1,034 ng/g (Fig. 1). In one patient, the concentration of drug in the tumour was significantly higher than that in normal breast tissue ($P < 0.001$). Tumour: normal drug concentration ratios for a single patient ranged from 1.27 to 8.36. No doxorubicin metabolites were detected in either type of tissue.

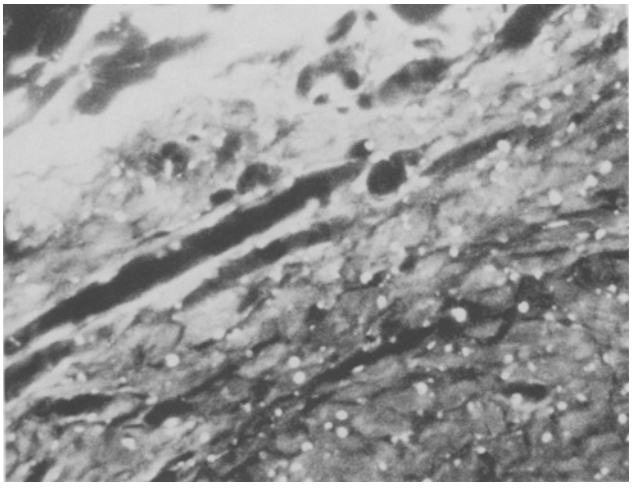

Fig. 4. Fluorescent staining of doxorubicin in a section of normal breast tissue (×10)

As an estimate of the relative cellularity of tumour and normal tissue, the total DNA content of both was assayed. When doxorubicin concentrations in tumour and normal tissue were expressed in such terms, there was still wide inter-patient variation in tissue drug concentrations in both tumour (range, 128–1,247 ng doxorubicin/mg DNA) and normal breast tissue (range, 205–967 ng doxorubicin/ng DNA) (Fig. 2). However, in one patient there was no significant difference between the drug concentrations in the tumour and those in normal breast tissue.

Serum drug concentrations

Linear correlation co-efficients were calculated for the relationship between tumour doxorubicin concentrations and serum parameters. There was a significant correlation between the 60-min tumour concentration and peak serum values ($r = 0.76$, $P < 0.05$) (Fig. 3). No doxorubicin metabolites were detected in serum.

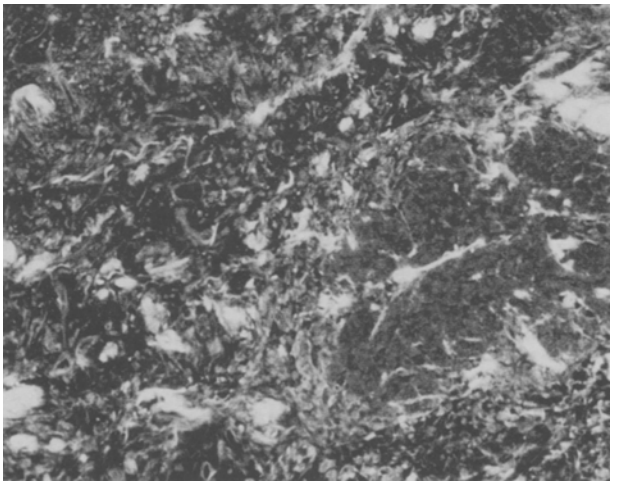

Fig. 5. Fluorescent staining of doxorubicin in a section of breast-tumour tissue (×10)

Relationship to other prognostic factors for breast cancer

No relationship was found between tissue doxorubicin concentration and differentiation state and either histological grade or oestrogen receptor status.

Fluorescence studies

Doxorubicin was distributed homogeneously in all sections of normal breast tissue (Fig. 4). There was also relatively intense fluorescent staining throughout all sections of breast tumour (Fig. 5). No drug-related fluorescent staining was seen in specimens from untreated control patients. The 30 fluorescence quantification readings from the four tumour sections ranged from 12 to 100 units of fluorescence (means, 35, 41, 47 and 37 units, respectively). No significant difference was found between the total fluorescence measured in the four tumour sections; however, there were marked differences in the degree of homogeneity of staining. Therefore, all sections were independently viewed and arbitrarily graded by two observers for homogeneity of staining (where 1 was the least homogeneous and 6 were the most homogeneous). There was no correlation between homogeneity of staining and quantities of drug measured.

Discussion

Early studies on the distribution of doxorubicin in animal models showed this drug to be extensively and rapidly taken up by all tissues of the body [2, 3, 26]. Although doxorubicin rapidly equilibrates with tissue extracellular fluid, from there it diffuses more slowly into cells. At physiological pH it is largely non-dissociated, lipid-soluble and capable of passing freely across cell membranes to bind reversibly to nuclear DNA. Serum pharmacokinetic studies in patients illustrate a bi-phasic plasma elimination curve, also consistent with retention of drug in peripheral tissues, followed by gradual drug release.

Some types of human tumour have been shown to take up relatively more doxorubicin than others, and uptake has been correlated with the expected clinical response rates for the particular tumour [9]. This result lends support to the idea that intra-tumour drug concentrations are relevant to response.

Few studies have attempted to correlate cytotoxic plasma drug levels in patients with the levels found within a tumour at a fixed time after drug administration [21]. It is assumed that a series of equilibria exist that relate free drug in plasma to drug at its site of molecular action within the tumour. In the present study, the wide range of patient variability in tissue drug levels can partly be explained on the basis of the pharmacological model, as there was a significant correlation between the patients' peak plasma and tumour tissue concentrations.

Although there is evidence from some types of tumour that specific drug-penetration barriers exist [20], it is generally assumed that tumour drug concentrations equilibrate with concentrations of drug in the tumour-bearing organ, down a diffusion gradient, and that this, in turn, is related to such factors as organ blood flow and tissue drug affinity. However, few studies have attempted to define this relationship [14].

We initially showed that tumour doxorubicin concentrations were apparently higher than those in normal breast tissue. The fact that doxorubicin has an affinity for nuclear DNA suggests that relatively more cellular tissues, particularly those with many dividing cells, would bind a greater quantity of doxorubicin than less cellular tissues. When doxorubicin concentrations were expresed in terms of tissue cellularity (using DNA content as a rough estimate of such), we found no significant difference in the concentrations of drug in tumour and normal breast tissue. This suggests that although the weight: weight tissue drug concentrations were different, cell: cell drug concentrations were similar. In breast cancer this difference is likely to be particularly marked, as the normal breast tissue, supporting stroma and fat have relatively few cells, especially in elderly women. A possible problem with this approach could arise from the fact that tumour cells may be aneuploid. The DNA content of tumour tissue may therefore not exactly reflect cell number, and an assessment of ploidy would also have been useful.

The absolute amount of drug present is one aspect of drug delivery to a tumour; the distribution of drug within the solid mass is also relevant. We used the autofluorescent properties of doxorubicin to examine the latter and found intense staining throughout normal breast tissue and tumour sections. The tumour sections showed variability in the intensity of staining, implying that certain areas within a tumour are exposed to more drug than others. Our initial studies using immunofluorescent staining for endothelial factor VIII, a marker of vascular patterns, suggests that the areas with increased fluorescent staining are those with more blood vessels (unpublished data).

Therefore the present data show that absolute drug uptake by the tumour and distant, normal breast tissue is similar at 1 h following the injection of a small dose of doxorubicin. Our results indicate that the amount of drug in the tumours correlate with peak serum doxorubicin concentrations in an individual patient. We also found that there were no gross penetration barriers to doxorubicin in breast cancer, but that the intra-tumoural distribution of drug was heterogeneous. The fact that only about 50% of breast cancer patients respond to doxorubicin suggests that mechanisms apart from drug delivery itself may be involved in determining response; these may include, for example, differing mechanisms of drug activity on a cellular level.

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