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Phenotypic variations dictate the intracellular compartmentalization of doxorubicin in normal human bone marrow cells

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Summary. In vitro accumulation of doxorubicin in intracellular compartments of normal bone marrow cells was studied with the use of fluorescent microscopy. Both the cytoplasmic and nuclear compartments had distinguishable drug accessibility in the diverse hemopoietic series and in different stages of maturation of each lineage. Nuclei appeared to be more sheltered in the myelogranulocytic series than in the nucleated erythroid cells. Nuclei of activated phagocytic cells of the myelogranulocytic series and macrophages appeared to be the least accessible to doxorubicin uptake. These observations establish that phenotypic variations dictate the patterns of anthracyclines' subcellular compartmentalization. They also suggest that the molecular mechanism contributing to the intracellular trafficking of doxorubicin deserves more substantial investigation that may contribute to our understanding of drug resistance and sensitivity.

Introduction

Doxorubicin (DOX) is a broad-spectrum chemotherapeutic agent with a major role in the treatment of leukemias, lymphomas, and solid tumors [19]. However, the use of this drug is limited by its cardiotoxicity, its suppression of hematopoiesis, and the surge of resistance. DOX is an anthracycline aminoglycoside that can intercalate into DNA, and such an interaction has been suggested as one of the major modes of its cytotoxic action. Additional subcellular targets might include the cell membrane, specific enzymes such as topoisomerase II, and mitochondria. Other mechanisms for cytotoxicity involve the quinone-mediated generation of superoxide radicals [4].

Uptake of doxorubicin by mammalian cells is thought to be due to passive diffusion through the plasma membrane [3]. No saturable, specific carriers were observed in studies using photoaffinity labeling of rat sarcoma plasma membranes [18]. The rate of uptake is temperature-dependent and influenced by the lipid composition of the membrane [14].

Drug resistance to anthracyclines has been extensively studied [2]. According to studies reported thus far, several cellular mechanisms appear to be involved; these may include an ATP-dependent efflux mechanism [8], altered membrane composition, elevated enzyme levels that lead to the destruction of superoxide radicals [1], and the appearance of modulated topoisomerase II [5, 6]. An altered intracellular compartmentalization in DOX-resistant leukemia cells has also been reported [13, 16, 17]. In most of the uptake and efflux studies, isotope-labeled anthracyclines or cytofluorometry were used on established cell lines [10, 12]. However, the kinetics of intracellular distribution and compartmentalization in normal cells as a function of cellular phenotypes has not yet received adequate investigation. The purpose of this study was to examine the kinetics of DOX uptake in normal human bone marrow cells by using fluorescent microscopy to monitor the drug's intracellular localization.

Materials and methods

Seven normal bone marrow specimens from normal bone-marrow transplant donors were drawn into preservative-free heparin solution and processed on the same day. Cells were separated from erythrocytes by the Ficoll-Hypaque technique. Cells from the interphase were washed two times with RPMI-1640 culture media and resuspended in the same media supplemented with 20% fetal bovine serum (heat-inactivated). Trypan blue exclusion was used to assess cell viability. Between 2 and 5 million cells were incubated with 1 μM DOX (Adria Laboratories, Columbus, Ohio) at 37° C in an incubator containing 5% CO_2/air. At different intervals, samples were removed and examined under oil immersion using a Leitz Dialux fluorescent microscope equipped with an HBO 50 mercury lamp and an N-2 filter system (excitation, 530–560 nm; long pass barrier, 580 nm). The location of DOX fluorescence in the plasma membrane, intracellular organelles, and nucleus was evaluated in 100 cells. Cellular identity was established by morphology under phase-contrast microscopy and confirmed by Giemsa staining.

Results and discussion

Bone marrow cells were examined with fluorescent microscopy after incubation with DOX for 5 and 30 min and 2, 4, 6, and 20 h (Table 1). Mature erythrocytes remained fluorescent-negative throughout the study.

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Table 1. DOX uptake by normal human bone marrow cells as determined by the intensity of DOX fluorescence in different subcellular compartments after incubation for different intervals

Cells	Intervals after addition of DOX				
Erythroid series:	30–60 min	2 hr	4 hr	6 hr	20 hr
Mature RBC	–	–	–	–	–
Reticulocytes (ZC)	–	++	++	++	++
Mature NRBC:					
Cytoplasm	–	–	–	–	–
Nuclei	+++	+++	+++	+++	+++
Immature normoblasts:					
Cytoplasm	–	–	+	+	+
Nuclei	++	++	+++	+++	+++
Granulocytic series:					
Cells + pseudopodia[a]	–	–	–	–	–
Promyelocytes, myelocytes:					
Cell membrane	+	+	+	+	–
Cytoplasm	+	+	+	+	–
Nuclei	–	++	++	++	++
Lymphocytes:					
Golgi apparatus	–	–	++	++	++
Nuclei	–	–	++	++	++
Macrophages[c]	–	–	–/+	–/+	–/+
Megakaryocytes:					
Cytoplasm	–	–	–/+	+[b]	+[b]
Nuclei	–	–	–	+	++
Platelets (ZC)	–	+	++	++	++

ZC, zone central; NRBC, nucleated red blood cells
[a] Negative in all subcellular compartments
[b] Positive granular distribution
[c] In some macrophages, after 20 h incubation fluorescent nuclei were observed that had been phagocytized

Young reticulocytes, which were distinguished by their special, folded morphology under phase-contrast microscopy, showed small, brightly fluorescent granules only at the hilar zones after 2 h incubation. This pattern did not change after 20 h incubation. The fluorescent granules may represent mitochondria or pockets in membrane folding. These areas are unlikely to adsorb DOX due to RNA-polysome complexes, since this drug does not readily intercalate into RNA. Furthermore, these reticulocytes are free of vacuoles; thus, DOX would not be trapped in such organelles. The virtual absence of detectable fluorescence in mature erythrocytes is of interest because it shows that their membranes are less accessible for DOX accumulation than the membranes of leukocytes. Only at exceptionally high drug concentrations (100 μM) is binding to spectrin observed [11].

The most remarkable finding was the rapid accumulation of DOX in the nuclei of nucleated erythroid cells (Fig. 1). Shortly after 30–60 min incubation, the nuclei of all of these cells were brightly fluorescent, whereas the nuclei of granulocytic and lymphocytic series were only faintly positive in 5%–10% of the cells. Late normoblasts appeared to be predominantly positive in the nuclei, whereas cytoplasmic organelles such as mitochondria or even the plasma membrane remained mostly negative after 6–20 h incubation. The rapidity with which DOX accumulated in the nuclei of these cells indicates that, as opposed to the situation in some granulocytic cells, there is no mechanism to shelter this compartment from DNA chelators. Only in the immature, large erythroblasts was a faint cytoplasmic fluorescence observed to contrast with the bright, clumped nuclei, which suggests that subcellular organelles are accessible for DOX accumulation.

Cells of myelogranulocytic series that were actively emitting pseudopods were distinguished by the absence of nuclear fluorescence. During the first 6 h incubation their nuclei remained negative; only faint plasma-membrane staining was observed. After 20 h incubation, the nuclear periphery was faintly visible in 40%–50% of the cells. The appearance of fluorescence at the nuclear periphery was similar to that in macrophages that were in active phagocytosis. The cytoplasmic portion of the pseudopods remained completely negative throughout the 20-h incubation, which suggested the exclusion of organelles capable of DOX accumulation. However, despite the lack of detectable fluorescence in the nuclei of some macrophages, after 20 h incubation one could observe in their cytoplasm fluorescent debris from other cells and fluorescent nuclei that had phagocytized from lysed, nucleated erythroblasts. Faintly positive fluorescence in the cell membrane and a few bright cytoplasmic granuoles were observed in promyelocytes and myelocytes after 2–6 h incubation. Positive, clumped chromatin that gave the nuclei a patchy appearance was also visible (Fig. 1). After 20 h incubation, the nuclei became more homogeneously positive. The fluorescence of plasma membrane and granules diminished, suggesting a shift of DOX to the nuclear compartment. At this time, 80% of the cells were still viable.

The limited DOX uptake in phagocytic macrophages and mature granulocytes with extending pseudopods is intriguing, especially since the cytoplasm of some macrophages contained nuclei engulfed with the accumulated

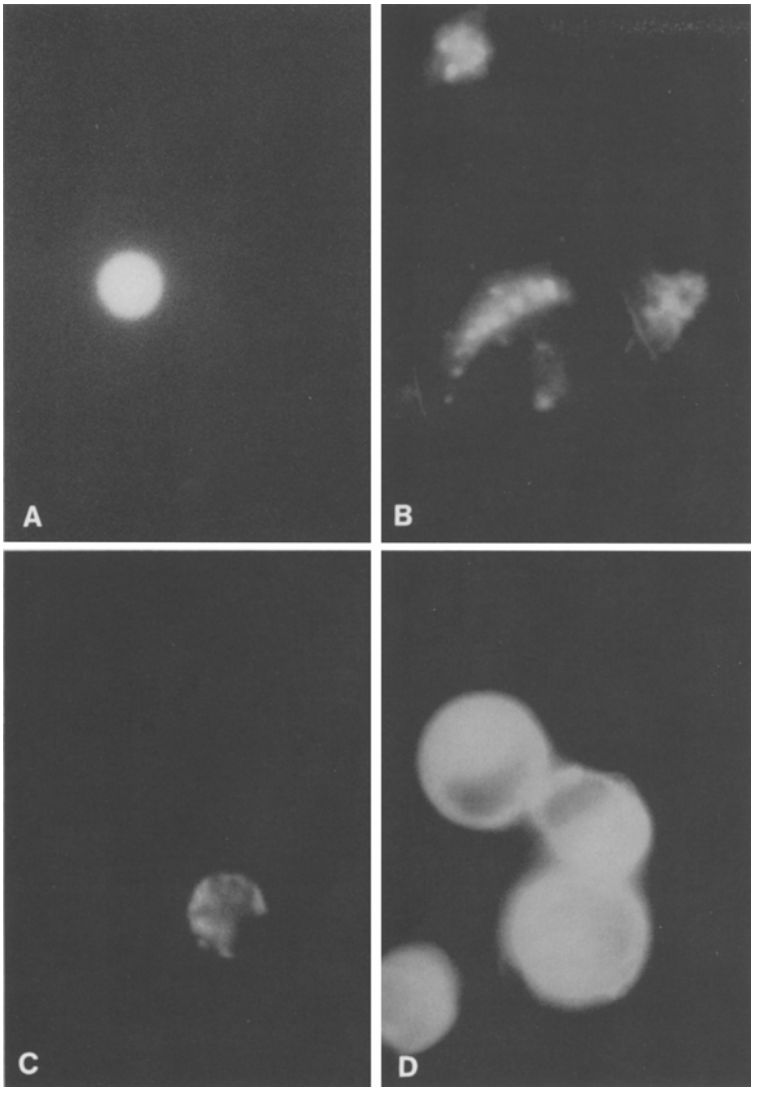

Fig. 1. DOX fluorescence in normal human bone marrow cells after incubation with 1 μM drug. **A** Intense nuclear staining of an erythroid cell after 30 – 60 min incubation. Note the lack of fluorescence in the plasma membrane and in subcellular organelles. This pattern did not change after even 20 h incubation. **B** Perinuclear granular staining pattern of a macrophage after 4 h incubation. The nucleus shows only faint staining in the center. **C** Patchy fluorescence in the nucleus of a promyelocyte after 2 h incubation. The plasma membrane is virtually negative, but after 6 h incubation a faint staining is visible. **D** After 20 h incubation, lymphocytic cells show uniform, intense staining in their nuclei and plasma membranes. No such pattern was observed in granulocytic cells

drug. Using flow cytometric techniques, Nooter et al. [12] reported diminished uptake of DOX by granulocytes as compared with lymphocytes in a subpopulation of rat bone marrow cells. In their report the stage of cell differentiation in the granulocytic series was not specified. We raise the possibility that either the granulocytes possess a strong efflux mechanism that is not available to erythroblasts during their functional stage or the changes in the cell membrane during phagocytosis make the cells less permeable to the drug. Although previous studies have established that DOX inhibits phagocytosis in macrophages and polymorphonuclears (PMNs), the authors did not elaborate on the drug's intracellular distribution [7, 15]. DOX also has been documented to increase spontaneous monocyte-mediated cytotoxicity [9]. Further studies are warranted to test the intracellular compartmentalization of DOX in resting, activated, and stimulated cells, as well as to test its effect on cell function.

Fluorescence began to appear after 4 h as a few bright spots near the nuclear indentation in small mononuclear cells with lymphocyte morphology. The nuclear periphery gradually became visible, and by 20 h all of the nuclei were positive. The positive spots, whose location and morphology was consistent with that of the Golgi apparatus [12], remained brightly fluorescent, whereas the rest of the cytoplasm remained remarkably negative. In DOX-resistant L-1210 lymphocytic leukemic cells, these patterns were very prominent, whereas the nuclei were virtually negative [13].

Cells with a morphology characteristic of megakaryocytes could be well distinguished in only two bone marrow samples. During 2- to 6-h incubations, the polylobated nuclei remained negative or only faintly positive; only after 20 h incubation were they intensely fluorescent. Positive granular cytoplasm was clearly visible. Thrombocytes separated from bone marrow by Ficoll-Hypaque gradient showed fluorescent granulation after 6–20 h incubation. Peripheral blood thrombocytes were also studied, but no granular pattern was observed. Differences in age and in ATP levels in platelets may account for this discrepancy. The uptake and efflux of DOX in these nuclear-free particles offer an interesting model for drug accumulation.

In summary, a marked difference in the nuclear accumulation of DOX was noted as a function of cellular differentiation. Intracellular drug distribution varied in cytoplasmic and nuclear compartments, depending on lineage and maturation, suggesting the existence of molecular mechanisms for DOX distribution to various compartments. These mechanisms might be similar to those utilized by resistant malignant cells. In view of the recent discovery of the P-glycoprotein as an efflux-related membrane component that correlates with multiple-drug resistance, it would be interesting to see if such a mechanism exists in normal bone marrow cells for the selective compartmentalization of DOX or to shelter the nuclear environment.

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