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Daunorubicin Reductase Activity in Human Normal Lymphocytes, Myeloblasts and Leukemic Cell Lines*

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Abstract—*To exploit the full potential of daunorubicin chemotherapy, it is necessary to understand its metabolism. We have shown previously that daunorubicin reduction in human liver is mediated by both aldehyde and ketone reductases. This study shows that this is also the case in normal blood cells. However, myeloblasts from AML patients show different pH profiles from those observed for normal lymphocytes. Human myeloid cell lines (KG1, ML1 and K562) accurately reflect the reductase heterogeneity seen in AML patients. This is in contrast to L1210 and P388 murine cell lines, which do not readily metabolize daunorubicin. When studying daunorubicin metabolism, it is important to use only cell lines that metabolize the drug because daunorubicin is extensively metabolized to daunorubicinol in AML patients. The use of human rather than rodent cell lines may provide useful information to increase our understanding of the* in vivo *situation.*

INTRODUCTION

ANTHRACYCLINE antibiotics are effective antineoplastic agents that show selective cytotoxicity against breast carcinoma, solid tumors and acute leukemia. Daunorubicin (D_1) is a ketone-containing anthracycline, and reduction of its carbonyl appears to be catalyzed by a class of drug-metabolizing enzymes in the cytoplasm of mammalian cells [1-3]. This class of enzymes includes aldehyde reductases (E.C. 1.1.1.2.), which catalyze the reduction of xenobiotic and naturally occurring aldehydes and aldoses, and ketone reductases, a similar yet distinct group of enzymes that appear to be specific for xenobiotic ketones.

We have shown that human and rabbit liver contains multiple D_1 reductases [4-6]. In addition to the pH 8.5 aldehyde reductase [3], we identified a new class of anthracycline antibiotic reductases not found in rat or mouse liver. This new reductase activity has an acidic optimum (≈pH 6.0) and is clearly distinguishable from the pH 8.5 D_1 reductase by ion exchange, gel filtration chromatography, isoelectric focusing and use of inhibitors [4, 5]. We have shown that the pH 8.5 D_1 reductase is inhibited by barbital but not pyrazole, clearly distinguishing it from pyrazole-sensitive, barbiturate-insensitive alcohol dehydrogenase and from the pH 6.0 D_1 reductase, which is insensitive to both barbital and pyrazole [4].

It is clear, therefore, that D_1 reduction to daunorubicinol (D_2) in human or rabbit liver is mediated by at least two classes of enzymes [4, 5], the pH 8.5 D_1 reductase activity, which is classified as an aldehyde reductase [3, 7, 8], and the pH 6.0 reductase activity, which is classified as a ketone reductase [4-6]. This indicated to us a complexity that was not originally appreciated when reductase activities were determined at pH 7.4. Because variations in either class of reductase could influence the overall metabolism of D_1 and subsequently chemotherapeutic response, we have evaluated the presence of different reductases in normal and myeloid leukemic cells and cell lines.

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MATERIALS AND METHODS

Daunorubicin was from Ives Laboratories, Inc., New York, and NADPH was from Sigma. Normal blood samples were from Lifeblood, Inc. and leukemic samples were obtained from newly diagnosed AML patients. Silica gel plates with fluorescent indicator were from Eastman Kodak Co. All other reagents and solvents were of the purest grade available.

Cell lines were maintained in continuous suspension culture in different media: ML1 cells in RPMI 1640, KG1 cells in αMEM and K562 cells in DMEM. All cell cultures were supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 in air. Cells were passaged twice weekly.

Cell preparation

Lymphocytes, myelocytes and erythrocytes were separated from blood and myeloblasts were separated from bone marrow by zonal density centrifugation with sodium metrizoate [9]. Contamination by erythrocytes in the lymphocyte and myeloblast preparations was easily eliminated by hypotonic lysis with the addition of 0.25% saline for 5-30 sec followed by washing with isotonic saline [9].

Enzyme preparations

All solutions for enzyme preparations were maintained at 0-4°C. Lymphocyte, erythrocyte and myeloblast cells were sonicated 4 times (10 sec each) in 0.002 M Tris-HCl, pH 7.4, using a Branson sonicater at a setting of 5 and a direct current of 4 A. This type of homogenate served as the source of enzyme activity for all experiments.

Daunorubicin reductase activity

The reaction mixture, in a final volume of 1.0 ml, contained 0.5 mM NADPH, 0.65 mM daunorubicin and buffers as indicated. The reductases were studied under optimal conditions and with linear kinetics. The reaction was initiated by addition of the enzyme and run at 37°C; background fluorescence was determined in the absence of NADPH and subtracted from that in the presence of the antibiotic. D_1 and D_2 were separated with $CHCl_3$:CH_3OH:H_2O (80:20:3; v/v/v) on silica gel plates (250 μm with fluorescent indicator) that had been activated at 120°C for 30 min before application of the samples [10, 11]. After chromatography the fluorescent areas were detected by ultraviolet light, scraped from the plates into test tubes and the fluorescent material eluted from the silica gel with 2 ml of 6.54 N H_2SO_4 in 95% ethanol. The concentrations of D_1 and its metabolite were determined using a Perkin-Elmer luminescence spectrometer (excitation wave length of 470 nm and emission spectrum at 585 nm) and a D_1 standard curve.

Determination of pH profile

The pH profile of D_1 reductase was determined in different buffers at concentrations giving conductivity readings of 8500-9500 mΩ. Buffers used were citrate phosphate (pH 5.0-6.3), potassium phosphate (pH 6.3-7.5), Tris-HCl (pH 7.5-8.5) and Tris-glycin (pH 8.5-9.5). Otherwise, assay conditions were identical to those described above for D_1 reductase activity.

Isoelectric focusing

Isoelectric focusing (IEF) was performed as previously described [4,5,12] using an LKB 8101 electrofocusing column (100 ml capacity) with 1% carrier ampholyte (pH range, 3-10) and a stabilizing sucrose gradient containing 0.5 mM dithiothreitol. Samples were introduced into the middle of the sucrose gradient of the column and electrofocusing lasted about 24 hr at 4°C with an initial power of 5.0 W. After electrofocusing, the column contents were collected in 1-ml fractions at a flow rate of 60 ml/hr; pH measurements and assays of D_1 reductase activities were performed immediately.

RESULTS

The D_1 reductase profile of normal human lymphocyte homogenate was found to have two distinct optima, one at ≈pH 6.0 and another ≈pH 8.5 (Fig. 1). This profile, which was similar to those of human and rabbit liver preparations [4, 5], suggests that daunorubicin reduction in human lymphocytes may be mediated by both

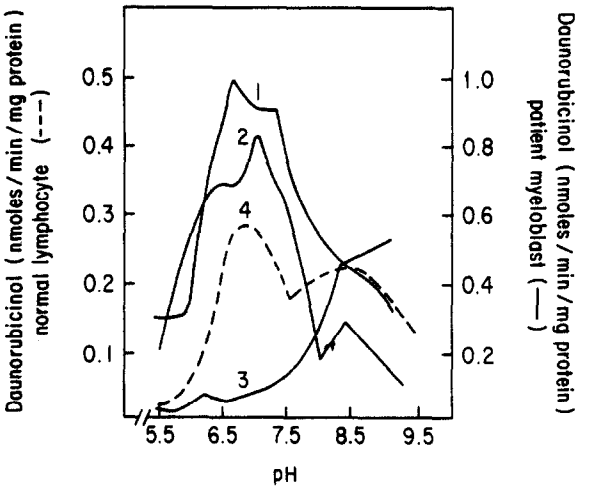

Fig. 1. Activity pH profile of daunorubicin reductase from normal human lymphocytes and from myeloblasts of three AML patients (at diagnosis): 1, N.R.; 2, M.M.; 3, K.L.M.; 4, normal lymphocyte.

ketone (pH 6.0) and aldehyde (pH 8.5) reductases. Lymphocytes had higher levels of both activities than myelocytes, which in turn had higher levels than erythrocytes. The ratio of pH 6.0:8.5 reductase activity, however, was very similar for all three cell types, suggesting that the two classes of enzyme are present normally in similar relative concentrations.

When we examined the pH profiles of enzyme preparations of myeloblasts from AML patients at diagnosis and before therapy, not only were the profiles quite different from those of normal lymphocytes, they were very different from one patient sample to another (Fig. 1). Each myeloblast preparation had its own characteristic pH profile, and each profile indicated that one class of enzyme was present in a much higher concentration than the other. Myeloblasts from patient N.R., for example, had mainly the pH 6.0 activity, with a low shoulder of pH 8.5 activity, while those from K.L.M. had almost no activity at pH 6.0, but substantial activity at pH 8.5. The pH profile of myeloblasts from patient M.M. was somewhat similar to that of normal lymphocytes, but the lower pH optimum was shifted to ≈7.0 and the ratio of the two activities was ≈2.8, compared to 1.0 in normal myelocytes and 1.2 in normal lymphocytes. When the myeloblast homogenate from patient K.L.M. was analyzed by IEF, no pH 6.0 reductase activity was detected and one peak of pH 8.5 reductase activity was observed (data not show), confirming that the bulk of reductase activity in this patient was associated with the pH 8.5 reductase class. However, normal lymphocyte homogenate contained both classes of reductases (Fig. 2) in addition to minor peaks of activity. Similar heterogeneity was previously observed in human and rabbit liver preparations [4, 5]; therefore it is likely that multiple forms of the reductases exist within each class.

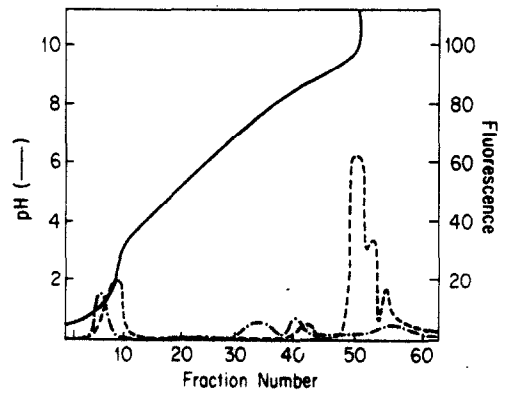

Fig. 2. Isoelectric focusing of human lymphocyte homogenate over a pH range of 3–10. Daunorubicin reductase activity at pH 6.0 (---) and 8.5 (— · — ·).

We have examined several human myeloid leukemic cell lines in our studies. The K562 cell line is composed of undifferentiated blast cells [13], the KG1 cell line is composed predominantly of myeloblasts and promyelocytes and the ML1 cell line is myelocytic. The K562 cell line exhibited low or no metabolism of $D_1 \rightarrow D_2$, suggesting very low levels of reductase activity (Fig. 3A). The KG1 cell line exhibited mainly the aldehyde reductase activity (pH 8.5), with little activity at pH 6.0 (<5%). The ML1 cell line exhibited both ketone and aldehyde reductase activity (Fig. 3A). When daunorubicin metabolism in human myeloid cells was compared to that of murine tumor cell lines such as P388 and L1210, no metabolism was detected at pH 5.0–7.5. Slight activity was detected for P388 cells at pH 8.5, while that of L1210 cells was <15% that observed with KG1 and ML1 cells (Fig. 3B).

When the cytotoxicity of D_1 was studied in human myeloid cells [13], the IC_{50}s (Fig. 4) were higher in the cell lines that do not metabolize $D_1 \rightarrow D_2$ as compared with those having one or both forms of reductases. The IC_{50} for D_1 in K562 cells was 17 nM, as compared with 10 nM in ML1 cells and 13 nM in KG1 cells. This suggests that K562 cells are more resistant to D_1 because of the lack of reductases.

DISCUSSION

If we are to realize the full potential of daunorubicin as a chemotherapeutic agent against leukemia, it is necessary to identify and understand the enzymatic mechanism(s) of its metabolism. Two main reactions take place. One

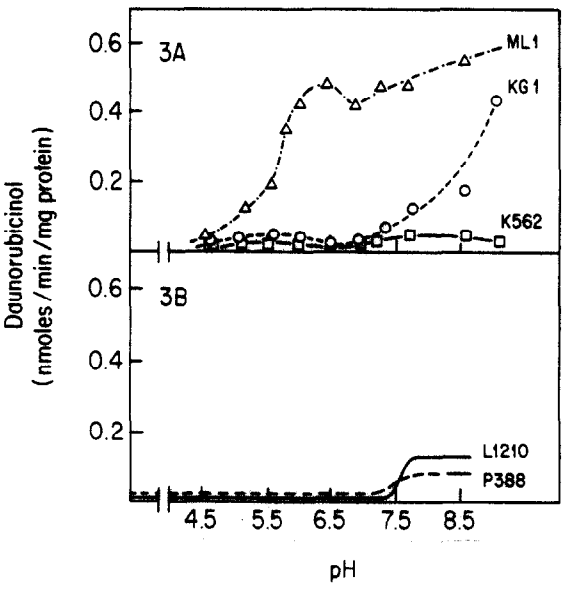

Fig. 3. Activity pH profile of daunorubicin reductase from different myelocytic cell lines.

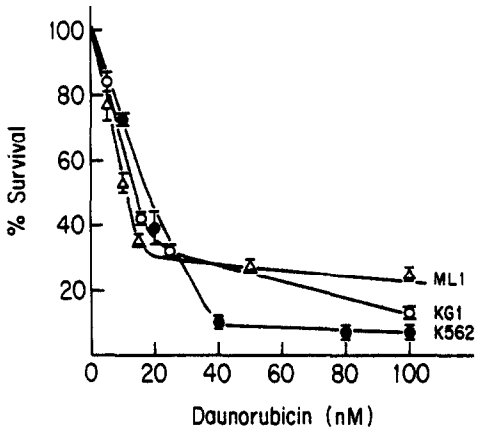

Fig. 4. Growth inhibition of ML1 (Δ——Δ), KG1 (○——○) and K562 (●——●) leukemic cell lines by exposure (72 hr) to daunorubicin.

is the reduction of the side-chain carbonyl group to a secondary alcoholic group. The reaction is mediated by both aldehyde and ketone reductases and the product is D_2. The second reaction is the reductive deglycosidation giving rise to deoxyaglycones. The semiquinones are formed as intermediates by a variety of enzymes (flavoenzymes) known to be able to accomplish one-electron reduction. These enzymes include reductases such as NADPH cytochrome P-450 reductase, nitrate reductase and NADH cytochrome reductases. Also, other enzymes, such as xanthine oxides and certain dehydrogenases, catalyze the same reaction.

With the cleavage of the glycosidic bonds by reductive or hydrolytic glycosidases [14], the aglycones are liberated in a deoxy or hydroxy form. However, D_1 aglycone is a product of *in vitro* mammalian metabolism [9] and is not found in human urine.

The main species present in bile are D_1 followed by D_2 and much smaller amounts of the aglycone type compounds. No aglycones have been reported in plasma or tissues *in vivo*.

Because the conversion of D_1 to D_2 *in vivo* seems to be the major pathway for D_1 metabolism, we have studied its metabolism via the reductases in both murine and human leukemic cell lines. Clearly D_1 is metabolized in human myeloid cell lines and myeloblasts from AML patients but not in rodent cell lines. Chemotherapeutic response depends on several dynamic factors: uptake, metabolism and efflux. It is important, therefore, to test these parameters in an *in vitro* model that is as analogous as possible to the *in vivo* situation. For example, the anthracyclines *in vivo* are subject to extensive metabolism [14, 15], but in experiments using rodent (rat or mouse) tumor cell lines *in vitro* [16, 17], no significant metabolism was demonstrated during short-term incubations. The antineoplastic activities of D_1 and D_2 have been compared using P388 leukemic cells *in vivo* [18]. When P388 tumor-bearing mice were treated once a day for 10 days with D_1 (1 mg/kg), the compounds had equivalent antileukemic activity. Importantly, D_2 retained the capacity of D_1 to inhibit nucleic acid metabolism [18, 19] and also had a longer half-life in both plasma and urine than did D_1 [20].

Because D_2 is cytotoxic and is retained longer by tissues than D_1, its intracellular production may favor cell kill. As has been shown in plasma from AML patients, concentrations of D_1 decline rapidly after a 4-hr infusion while the concentration of D_2 exceeded that of the parent compound only 5 min after the infusion [21]. Furthermore, D_2 concentrations in leukemic cells were 200-fold higher than plasma concentrations [22]. The drug is cleared rapidly from plasma and much of it is retained in leukemic cells which metabolize and excrete it slowly. Since D_2 is less lipophilic than D_1, its efflux from these cells can be expected to be much slower, resulting in selective leukemic cell kill.

Our results suggest that murine tumors are inadequate models for studying D_1 metabolism in humans because variations in, or absence of, one or both groups of enzymes could influence overall anthracycline metabolism.

A question to be addressed is whether or not metabolism is altered in drug-resistant cells. Possibly, in addition to altered uptake, efflux and membrane glycoprotein changes, metabolism is also altered. Most earlier studies of D_1 resistance were performed using murine tumor cell lines (e.g. P388, L1210, Ehrlich ascites, L5178Y). It is likely that the reported insignificant metabolism is due to the lack of the pH 6.0 D_1-activity and to the use of murine tumor cells that do not metabolize D_1 very well. We have developed resistant human myeloid cell lines and found that, with 50- and 150-fold resistant cells, metabolism was altered [unpublished observation].

When studying D_1 metabolism *in vitro*, therefore, it is important to use cell lines that are capable of metabolizing the drug. Because D_2 is more polar than D_1 and more easily retained by tissues [23], formation of the metabolite in leukemic cells may be an important consideration in the chemotherapeutic response to D_1. Furthermore, if we are to increase our understanding of normal anthracycline metabolism, cell lines should reflect D_1 metabolism in normal hematopoietic cells. Cell lines must be studied that are representative of the variability in reductase activity and D_1 metabolism in leukemia patients. Hence a cell line such as K562, that exhibits little

or no *in vitro* metabolism of D_1, is not an appropriate model system because the vast majority of patients do metabolize D_1. We will continue to use human cell lines that metabolize the drug as they provide a useful model system for studying the *in vitro* metabolism of D_1 in human myeloid leukemia. Currently, we are studying D_1 uptake, metabolism, retention and efflux in sensitive and resistant cells. These studies should provide useful information to improve our understanding of the *in vivo* metabolism of daunorubicin.

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