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Survival of rat mammary tumor cell clones and DNA strand damage following adriamycin treatment*

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Summary. Tumor cell subpopulations have been shown to be heterogenous in a number of phenotypic characteristics, including responses to cytotoxic drugs. This phenotypic heterogeneity has been used here to study mechanisms associated with Adriamycin (doxorubicin HCl)-induced cytotoxicity. Clonogenic survival and alkaline elution methods were employed to examine the response of two tumor cell subpopulations to Adriamycin. The cells were derived from a primary 13762NF rat mammary adenocarcinoma (clone MTC) and a lung metastasis in the same animal (clone MTLn3). The MTC cells were significantly more resistant to Adriamycin than were the MTLn3 cells; the dose effective in reducing cell survival by 50% was 10-fold higher. Protein-associated DNA strand breakage assayed by alkaline elution was dose-dependent in both clones, and MTC cells were again more resistant to break induction than were MTLn3. These results showed that clonal tumor subpopulations isolated from a primary tumor and its metastases possessed different intrinsic survival responses to Adriamycin treatment in vitro and that this survival response correlated with Adriamycin-induced production of protein-associated DNA single-strand breaks.

Introduction

Adriamycin (doxorubicin HCl) is a common antineoplastic drug used in the treatment of many cancers, including acute leukemia, non-Hodgkin's lymphomas, breast cancer, Hodgkin's disease, and sarcomas; yet, its mechanism of action is not clear [19]. There are several suggested mechanisms of action ascribed to the anthracyclines: first, intercalation with DNA, resulting in inhibition of repair [8, 15], replication [2], or transcription [2, 15]; second, protein-associated DNA single-strand breaks mediated by superoxide or hydroxy radical formation [13, 14, 16]; and, third, fragmentation of DNA [15]. However, a specific anthracycline-induced DNA lesion has not been identified, and the mechanisms of cytotoxicity are likely to be multiple [5, 11]. For example, investigators have shown that Adriamycin binds to the membrane at concentrations below those that affect DNA functions [19]. At these concentrations lipid peroxidation or changes in plasma membrane redox functions can occur [3, 4, 16].

As a test of the relationship of protein-associated DNA single-strand breaks to mechanisms of Adriamycin cytotoxicity, we used clonogenic survival and alkaline elution methods to correlate breaks with survival. These studies were done using clonal populations of the 13762NF rat mammary adenocarcinoma. In previous studies, clones derived from a locally growing tumor, MTC, and a lung metastasis, MTLn3, were found to differ in Adriamycin cytotoxicity, MTC cells being more resistant than MTLn3 cells at the same passage number [17]. We confirmed this heterogenous survival response and found that the order of sensitivity to Adriamycin-induced strand breaks was the same as the order of sensitivity to Adriamycin-induced lethality.

Materials and methods

Cell culture. The tumor cells used in this study were clones MTC and MTLn3, isolated from a primary 13762NF rat mammary adenocarcinoma and its lung metastases, respectively. Their cloning and characterization have been described previously [9]. Cells were grown at 37 °C in a humidified atmosphere (95% air and 5% CO_2) in 100-mm Corning culture dishes (Corning Glass Works, Corning, NY) containing alpha-modified minimum essential medium, AMEM, (GIBCO Laboratories, Grand Island, NY), 10% fetal bovine serum (Reheis Division, Armour Pharmaceuticals, Phoenix, Ariz), and no antibiotics.

Labeling and adriamycin treatment for alkaline elution. Four 60-mm culture dishes were plated with 3×10^5 cells for each tumor clone, and cells were labeled with 0.02 μCi [^{14}C]thymidine/ml AMEM for 24 h. They were then rinsed twice and the label chased with 0.2 μg "cold" thymidine/ml AMEM for 24 h. Next, cells were incubated with 0, 0.1, 5, or 50 μg Adriamycin/ml for 1 h, rinsed twice with calcium- and magnesium-free phosphate-buffered saline (2.7 m*M* KCl, 137 m*M* NaCl, 1.5 m*M* KH_2O_4, 8 m*M* Na_2HPO_4, pH 7.4) (CMF-PBS), and removed from the dishes with 1% trypsin. All solutions were kept at 4 °C to prevent repair of Adriamycin damage. Adriamycin was obtained from Farmitalia Carlo Erba (Milan, Italy) in

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10-mg vials, dissolved in 5 ml CMF-PBS, and used as a stock solution for subsequent dilutions.

Alkaline elution. Alkaline elution was used to measure the rate and amount of DNA single strands released in alkali that were able to pass through polycarbonate membrane filters. The alkaline elution procedure was modified from that described by Kohn et al. [6, 7]. In brief, approximately 1×10^6 cells from each Adriamycin treatment concentration were placed on polycarbonate membranes (2-μm pore size, 25 in diameter, Bio-Rad Laboratories, Richmond, Calif) that had been wetted with 5 m*M* EDTA in CMF-PBS. Cold lysis solution (69 m*M* sodium dodecyl sulfate and 36 m*M* EDTA) containing 0.5 mg proteinase K/ml was then added to the cells for 30 min. This treatment reduced or eliminated the effects of DNA-protein cross-links. After the lysis solution had been eluted by gravity, the DNA was rinsed with 20 m*M* EDTA, followed by addition of 35 ml alkaline elution solution (100 m*M* tetrapropylammonium hydroxide, 3.8 m*M* sodium dodecyl sulfate, and 20 m*M* EDTA, pH 12.1). The flow rate was 0.04 ml per min and 3.5-ml fractions were collected in the dark every 90 min for 15 h. The fractions, washes, and filters were counted using a scintillation counter, and the percentage of DNA left on the filter during the course of the elution was calculated. Three replicates were done for each concentration of Adriamycin and each tumor cell clone.

Survival studies. The cells were rinsed twice with warm CMF-PBS and detached with warm 0.25% trypsin. They were next resuspended in cold AMEM, and cell counts were then done using a hemacytometer. Appropriate cell dilutions were made in cold AMEM, and cells were plated onto 60-mm culture dishes containing AMEM. Cells were incubated 12–14 h at 37 °C to allow the cells to attach firmly to the dish and recover from trypsin effects. The medium was then aspirated from the cells and replaced with 3 ml prewarmed AMEM containing either no Adriamycin or Adriamycin at concentrations of 0.01, 0.05, 1.00, 2.00, 5.00, 10.00, or 50.00 μg/ml. After a 1-h incubation, the Adriamycin-containing AMEM was carefully aspirated, the cells were rinsed twice as described above for alkaline elution, and the medium was replaced with Adriamycin-free AMEM. The cells were then incubated for 8 days, after which time they were fixed with Carnoy's fixative (3 : 1 vol./vol. methanol:acetic acid), stained with crystal violet, and counted.

All colonies of 50 cells or more were scored as positive for survival. Percentage survival was calculated as described previously [18]. Three replicates were done for each concentration of Adriamycin and each tumor cell clone.

Results

MTC and MTLn3 cells were heterogenous in their clonogenic survival response to Adriamycin treatment, MTC being more resistant than MTLn3. The dose effective in reducing cell survival by 50% (ED_{50}) for MTC cells was approximately 0.16 μg/ml, whereas it was approximately 0.016 μg/ml for MTLn3, a factor-of-10 difference between these two tumor subpopulations. There was also a very large difference in the shoulder region of the survival curve, MTC requiring a much higher concentration to appreciably decrease surviving fraction than MTLn3 (data not shown). These survival results generally confirmed those previously reported by Welch and Nicolson from cryogenic stocks of the same tumor cell clones [17].

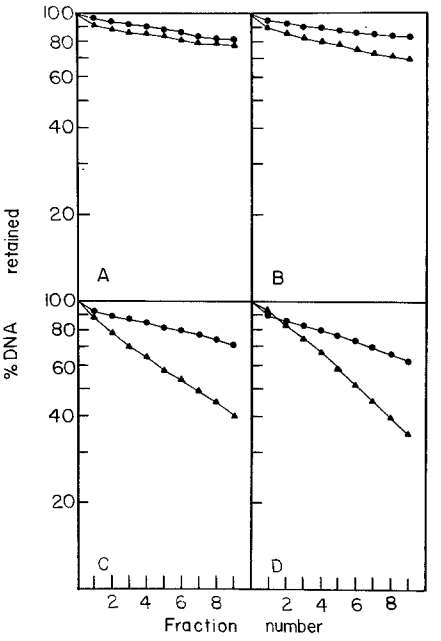

Fig. 1 A–D. Representative alkaline elution profiles of the MTC *(closed circles)* and MTLn3 *(closed triangles)* tumor cell clones not exposed to Adriamycin **(A)** or exposed to Adriamycin concentrations of 0.1μg/ml **(B)**, 5.0 μg/ml **(C)**, and 50 μg/ml **(D)**

This reproducible difference in relative Adriamycin cytotoxicity survival of the two tumor clones was compared with the difference between them in Adriamycin-induced DNA strand breakage (Fig. 1). The strand breakage was demonstrated to be protein-associated in separate experiments by eluting through polyvinyl chloride filters during alkaline elution in the presence or absence of proteinase K. In the absence of proteinase K, the levels of eluted DNA were dramatically reduced to approximately those of the controls (Evans et al., unpublished data). At all concentrations of Adriamycin assayed by alkaline elution (0.1, 5, or 50 μg/ml) there was a dose-response effect within each tumor cell clone and the MTC cells were more resistant than MTLn3 to Adriamycin-induced single-strand breakage. Thus, the order of Adriamycin sensitivity was the same as the order of sensitivity to Adriamycin-induced lethality in these two tumor subpopulations originating from the same tumor.

Discussion

Adriamycin is in common use for the treatment of many neoplasms, but its actual mechanism or mechanisms of action in cells are equivocal and probably complex. In the present work, the production of DNA single-strand breaks was found to be dose-dependent and to be associated at each dose with Adriamycin-induced cytotoxicity (Fig. 1) [17]. As previously reported by Zwelling et al. for L1210 cells [20], we found that Adriamycin produced approximately first-order elution kinetics for the percentage of DNA retained with respect to time for each drug concen-

tration in the two different tumor cell clones (Fig. 1). Further, these strand breaks were protein-associated; elution in the absence of proteinase K did not result in any detectable stand breaks.

The data also illustrated tumor cell heterogeneity: clonal tumor cell populations isolated from the same animal responded differently to the same treatment. The clone MTC may have been more resistant to Adriamycin-induced cytotoxicity owing to multiple mechanisms, which may be related to reduced drug uptake or increased efflux. Both mechanisms have been shown by others to occur in various cell lines [1, 12], but have not yet been studied in our clones.

Regardless of the mechanism(s) of resistance, the number of Adriamycin-induced single-strand breaks was reproducibly different and correlated with cytotoxicity. The relationship between strand breaks and cytotoxicity may differ slightly with other intercalators. Pommier et al. recently compared the intercalators 4'-(9-acridinylamino)methanesulfon-*m*-anisidide and 5-iminodaunorubicin for single- and double-strand DNA breaks, sister chromatid exchanges, mutations, and cytotoxicity in Chinese hamster V79 cells [11]. Although both induced-single- and double-strand breaks, the authors concluded that the double-strand breaks induced at DNA topoisomerase II-binding sites were more closely correlated with sister chromatid exchanges, mutations, and cytotoxicity. In prior studies that included the use of these two intercalators and Adriamycin in L1210 cells, this group concluded that intercalator cytotoxicity was not related to intercalator-induced DNA strand breaks [10, 21]. The difference in results between L1210 and V79 cells was not explained, but it may be a result of phenotypic heterogeneity.

There are clearly multiple mechanisms whereby Adriamycin intercalation can be modulated; however, the present results from two tumor subpopulations and those of others support the conclusion that intercalation results in protein-associated DNA strand breaks and that these breaks at least correlate with Adriamycin cytotoxicity.

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