Adriamycin: Protection from Cell Death by Removal of Extracellular Drug

Paul Vichi and Thomas R. Tritton

Vermont Regional Cancer Center [P. V., T. R. T.] and Department of Pharmacology [T. R. T.], University of Vermont College of Medicine, Burlington, Vermont 05405

ABSTRACT

Adriamycin is a cytotoxic drug which has enjoyed considerable success in the treatment of cancer. This agent has a bewildering variety of biological effects both within and on the surface of cells exposed to drug, and it has proved difficult to unambiguously assign a single mechanism of action. In this report we are able to separate intracellular and extracellular actions by taking advantage of the complete lack of Adriamycin-induced cytotoxicity at low temperature. For example, cells exposed to 100 \( \mu \)M Adriamycin at 0°C are not killed by the drug, even though this concentration is orders of magnitude higher than the concentration needed to cause 100% cell death at 37°C. If cells exposed to 100 \( \mu \)M Adriamycin at 0°C are shifted to fresh drug-free medium at 37°C, there is a time-dependent decrease in survival. However, if the drug-free medium contains calf thymus DNA (1.5 mg/ml) to act as a reservoir for Adriamycin binding of effluxed drug, there is no ensuing cytotoxicity. Thus, the results show that no matter how much drug is present inside the cell, there must also be extracellular drug available for membrane interaction in order to initiate nuclear DNA damage and the cytotoxic cascade.

INTRODUCTION

The anticancer drug Adriamycin causes a large variety of biochemical effects on exposed cells. For example, disruption of nuclear, cytoplasmic, and plasma membrane properties have all been advanced as contributing to the cytotoxic action of the drug (reviewed in Refs. 1–3). Consequently, a simple definition of the most important locus of performance has been elusive. Earlier work has demonstrated that the antiproliferative ability of Adriamycin is eliminated at temperatures below about 20°C and that this loss of activity is not due to alterations in uptake, biotransformation, or cellular distribution (4). Furthermore, Adriamycin appears to be unique in this respect. Drugs such as bleomycin, platinum compound ARK73-21, mitomycin C, and etoposide all retain cytotoxic activity at low temperatures (4). However, the temperature dependence of Adriamycin-induced DNA damage mediated by topoisomerase II parallels the temperature dependence of cytotoxicity: both cell death and DNA-protein cross-links are halted by lowering the temperature below 20°C (5). Thus DNA damage appears to be functionally linked to cytotoxicity, but there are at least two reasons to think that factors controlling the ability of cells to sustain DNA damage could be located at sites external to the nucleus: (a) By using Adriamycin fluorescence emission as a probe of its own membrane microenvironment (4), it can be shown that there is a structural change in the plasma membrane at 20°C; thus the membrane organization at the drug-binding loci changes at the same temperature as the loss of cytotoxic capacity, and it is possible that the corresponding shift in fluidity underlies the decoupling of a signaling process in the mechanism of Adriamycin-induced cell death. (b) Although DNA damage appears to be functionally linked to cytotoxicity in whole cells, investigations of DNA damage in isolated nuclei (5) reveal that removal of cytoplasmic and membrane components disrupts the DNA-protective mechanism operative in whole cells at low temperature. It is possible that Adriamycin induces DNA damage by a different mechanism in whole cells than in isolated nuclei, although we think this is unlikely since the types of damage sustained are qualitatively similar in both cases. Whatever the case, the previous results show that isolated nuclei do accumulate Adriamycin-induced DNA damage at low temperature suggesting that control of DNA damage in the intact cell resides at sites external to the nucleus. We can gain insight into this possibility by capitalizing on the temperature-sensitive nature of Adriamycin action, a property which allows us to isolate the effects of intracellular drug from the effects of drug interacting with the plasma membrane. The results show that a signal from the cell surface is required to initiate DNA damage in the nucleus and subsequent cell death.

MATERIALS AND METHODS

Growth of Cells. L1210 mouse leukemia cells were grown in suspension culture in McCoy's 5A medium with l-glutamine and 10% horse serum at 37°C in a 10% CO2 atmosphere. The cells were passaged twice weekly and recloned after every 50 to 60 passages.

Cytotoxicity Measurement. The effect of Adriamycin on cell growth was determined by cloning in soft agar at 37° as described previously (4). Cells in mid-log phase (2–5 \( \times \) 10⁵ cells/ml) were exposed to Adriamycin at a given concentration, time, and temperature in complete McCoy's 5A medium with l-glutamine and 10% heat-inactivated horse serum (Gibco). Following treatment, cells were centrifuged at 1500 rpm for 5 min at 4°C and the pellets were washed three times in cold, complete McCoy's 5A medium. The pellets were resuspended to a final concentration of 1 \( \times \) 10⁵ cells/ml and the cells were serially diluted into complete McCoy's 5A at ratios of 1:20 and 1:10. The entire 1:10 dilution (3 ml) was added to cloning medium consisting of 24.5 ml of complete McCoy's 5A with 15% horse serum and 2.5 ml of 5% Bacto Agar (Difco). The cells were then plated into 60-mm tissue culture dishes (Corning), 6 ml/plate. After the contents gelled, plates were transferred to an incubator at 37°C in 10% CO₂, and colony-forming efficiency was assessed by counting the number of clones formed after 10–14 days when seeding a known number of cells. The results shown thus represent the percentage of cells which survive a specified treatment condition.

DNA Damage. Nuclear DNA damage was quantified as single-strand breaks by alkaline elution as previously described (5). Growing cells were divided to 5 \( \times \) 10⁴/ml in medium containing 0.5 \( \mu \)Ci/ml of [³H]thymidine to label DNA and grown for 24 h in a CO₂ incubator at 37°C. The cells were then washed and grown for an additional 18 h prior to an experiment. After treatment with Adriamycin at specified conditions, the cells were washed, lysed in lauryl sarcosine and proteinase K on polycarbonate filters, and eluted under alkaline conditions. The frequency of single-strand breaks was calculated as described (5). Adriamycin Quantitation. Intracellular Adriamycin was analyzed in cells at pepsin by lysis with 1 ml of 2% Na₂CO₃ followed by extraction with successive additions of 2 ml of ethyl acetate:n-propyl alcohol (9:1). The organic layers from each extraction were pooled and evaporated under \( \text{N}_2 \) gas. Residues were resuspended in 2 ml of ethanol (70%), and Adriamycin content was quantified by fluorometric analysis on an SLM...
ADRIAMYCIN: AVOIDING CELL DEATH BY REMOVING EXTRACELLULAR DRUG

4800 fluorescence spectrophotometer by comparison to known standards. All measurements were performed in triplicate.

RESULTS AND DISCUSSION

In order to begin to establish which sites are essential for Adriamycin to induce loss of proliferative capacity, we first asked whether the cytotoxic process could be reversibly halted or initiated by temperature shifts. Fig. 1A shows that cells in the presence of high levels of Adriamycin do not suffer cytotoxic consequences at 0°C but immediately lose reproductive viability when shifted to 37°C. Thus, exposure to low temperature does not cause a permanent inability to respond to drug action. Similarly, if the drug exposure begins at 37°C, the time-dependent loss of clonogenicity is halted by lowering the temperature to 0°C (Fig. 1B). Therefore cells can be rescued from a cytotoxic process already in progress simply by lowering the temperature.

Fig. 1. Effect of temperature on Adriamycin toxicity. Data are represented as the percentage of survival of treated cells relative to untreated controls. A, reversal of low-temperature protection. L1210 cells were exposed to 100 μM Adriamycin for 2 h at 0°C. Aliquots were removed, washed, and cloned in soft agar. The remaining cells were shifted to 37°C for up to 4 h with samples being cloned at the indicated times. B, reversal of high-temperature cytotoxicity. L1210 cells were exposed to 0.05 μM Adriamycin for 1 h at 37°C. Aliquots were then removed, and survival was measured by cloning in soft agar. The remaining cells were either transferred to 0°C or maintained at 37°C for an additional 4 h. ☐, cells at 0°C; ☐, cells not washed.

Since the time course of cytotoxicity can be interrupted at any point, we realized that cells could be loaded with intracellular Adriamycin at low temperature, washed free of extracellular drug, and then observed for cytotoxicity at high temperature with drug initially present only in the interior. This scheme offers a way to ask whether the presence of intracellular drug is sufficient to cause toxic action. The issue is complicated by the fact that intracellular drug will be effluxed until a new equilibrium is reached; therefore, it becomes necessary to measure both intracellular and extracellular drug concentrations under various conditions. Fig. 2 shows the results of such experiments. If cells are exposed at 0°C to 5 μM Adriamycin the intracellular concentration achieved is 10 μM. However, if the extracellular drug is washed away there is no ensuing cytotoxicity (Fig. 2A) when the temperature is shifted to the permissive 37°C, even though the intracellular Adriamycin concentration is at least 4 times that necessary to achieve cytotoxicity when the drug is given directly at 37°C (Table 1). Thus, despite the fact that the cell has a relatively high level of Adriamycin inside, it does not suffer any toxic consequences.
The extracellular anthracycline concentration due to efflux under these conditions is $3 \times 10^{-9}$ M (Table 1); therefore, we reasoned that perhaps this level is not adequate to initiate cell membrane processes involved in cytotoxicity. To test this possibility we repeated the low temperature loading using a 20-fold greater (100 μM) Adriamycin concentration. After the external drug was washed away the intracellular concentration remaining was 40 μM (Table 1). Measurement of the extracellular concentration attained after shifting to 37°C for 2 h was $5 \times 10^{-8}$ M. Under these conditions there is now a time-dependent decrease in cell survival (Fig. 2B); cells exposed to 100 μM Adriamycin at 0°C but never shifted to 37°C show no decrease in survival no matter how long the exposure. An explanation for these results is that drug does have present on the exterior of the cells at 37°C in order to cause cytotoxicity, and the external concentration must exceed a threshold level of about $10^{-8}$ M. It would seem likely that any drug bound to the membrane could partition between both halves of the bilayer. Thus, our results imply that sites in or on the membrane that are available for binding when the drug approaches the cell from the outside are not available when the drug binds to the inner bilayer leaflet of the membrane via approach from the cytoplasm.

In the above experiments the source of extracellular drug is the reservoir inside the cells initially loaded at low temperature. To determine if this effluxed drug was truly required for cytotoxicity we repeated the experiment with a large excess of high molecular weight calf thymus DNA added to the external medium. Our reasoning was that this high-affinity and high-capacity binding site for Adriamycin would effectively remove any effluxed drug from further interaction with the plasma membrane and thus prevent the cytotoxic action. Fig. 3 shows that this prediction is borne out; no matter how high the intracellular concentration there is no growth inhibition by Adriamycin if the extracellular drug concentration is eliminated by DNA complexing. Furthermore, nuclear DNA damage (measured by alkaline elution) is also prevented by complexing the extracellular Adriamycin (Fig. 3B). Thus, in order to provoke cytotoxicity Adriamycin must be present in the extracellular milieu where it can interact with the cell surface. The process of drug exiting the cell is apparently not sufficient to signal cytotoxicity; if it were, external DNA would not provide protection. Instead, a membrane-mediated event appears necessary to initiate the cascade which eventually ends up in nuclear DNA damage and cell death.

What is the sequence of events which triggers the cytotoxic pathway? The accumulation of results imply that both DNA damage in the nucleus and drug perturbation of the plasma membrane are required for Adriamycin action. Since the DNA damage is catalyzed by topoisomerase II (6) we conclude that a signaling event at the surface is capable of controlling the activity of the nuclear enzyme. It is known that topoisomerase II can be covalently modified by serine/threonine (7, 8) and tyrosine (9) phosphorylation as well as by ADP ribosylation (10, 11); these enzymes thus represent potential levels of control. In earlier work we showed that Adriamycin treatment of cells increases phosphoinositide turnover, causing an accumulation of diacylglycerol and activation of protein kinase C (12). Since this serine/threonine kinase can use topoisomerase II as a substrate for phosphorylation (7), this pathway seems to be an attractive candidate for transducing signals from the plasma membrane to the nucleus, which ultimately give rise to cell death. Other signaling pathways may also be important and this possibility is under active investigation in our own and other laboratories.
ACKNOWLEDGMENTS

We thank Dr. Miles Hacker for reading the manuscript and Dr. James Posada, Dr. Alok Bhushan, and Dr. Elizabeth Dolci for their comments on the work.

REFERENCES

Adriamycin: Protection from Cell Death by Removal of Extracellular Drug

Paul Vichi and Thomas R. Tritton


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/52/15/4135](http://cancerres.aacrjournals.org/content/52/15/4135)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.