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Mechanism of action and spectrum of cell types susceptible to doxorubicin photochemotherapy

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Abstract We have shown that, whereas argon ion laser irradiation alone is not cytocidal for L929 cells, it greatly increases the cytotoxicity of intracellular doxorubicin. The present study showed that light enhancement of doxorubicin cytotoxicity was not restricted to stock L929 cells, but could also be demonstrated using L929 cells selected for doxorubicin resistance and several standard cell lines that are relatively resistant to doxorubicin prior to selection. Light-enhanced cytotoxicity resulted in extensive nuclear DNA loss and was strongly inhibited by anoxia. These findings suggest that the mechanism by which light exposure enhances doxorubicin cytotoxicity involves DNA damage by intranuclear generation of reactive oxygen species.

Key words Adriamycin · Doxorubicin resistance · Drug resistance · Free radicals · Photochemotherapy · DNA damage · Doxorubicin · Laser

Introduction

Photochemotherapy, in the context of cancer treatment, is generally considered to be a process which generates cytotoxic agents when incident light is absorbed by nontoxic compounds that are selectively taken up by tumor cells [2]. However, we have shown that the potential applications of photochemotherapy may be further expanded to include enhancing the effectiveness of already recognized cancer chemotherapeutic agents. These studies employed argon ion laser irradiation to enhance the cytotoxicity of doxorubicin toward cultured L929 cells [9] and demonstrated that clonogenic survival could be decreased by several orders of magnitude relative to doxorubicin alone.

Although L929 cells are a standard laboratory cell line, they are not directly comparable to any common human malignancy. Therefore, the present studies were undertaken to extend the initial observations to cell lines derived from human epithelial tumors and to gain additional insight into the mechanism of cytotoxicity enhancement. The findings indicate that two human epithelial cell lines tested as well as a line of doxorubicin-resistant L929 cells were susceptible to light enhancement of doxorubicin cytotoxicity. The mechanism of this effect appeared to be highly dependent on molecular oxygen and to involve a degree of DNA damage which was not produced by doxorubicin alone. The implications of this finding for the mechanism of conventional doxorubicin cytotoxicity are discussed.

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Materials and methods

Chemicals

Doxorubicin was obtained from Chiron Therapeutics (Emeryville, Calif.).

Cells and culture conditions

Murine L929 cells (a permanent fibroblast-related line), a human breast adenocarcinoma cell line (MCF-7) and a human bladder transitional cell carcinoma cell line (RT-4) were used. All cell lines were obtained from MA Bioproducts (Bethesda, Md.) and were routinely maintained in high-glucose Dulbecco's modified eagle's

minimum essential medium (DMEM) and 10% newborn calf serum (L929) or 10% fetal calf serum (MCF-7 and RT-4).

The doxorubicin-resistant cell line L929/DOX was prepared by treating the L929 cell line with gradually increasing concentrations of doxorubicin in vitro until the concentration of 50 μg/ml was attained. A resistant subclone was then treated once with 100 μg/ml of doxorubicin and the surviving resistant population used in subsequent experiments. The L929/DOX cells were maintained in DMEM medium supplemented with 10% fetal calf serum and lacking doxorubicin. Drug efflux experiments (data not shown) indicated that doxorubicin was pumped out of the resistant cells much faster than out of the wildtype cells, consistent with a multidrug resistant phenotype.

Cells from the stock cultures were plated in 14-mm multiwells or in 35-mm plastic tissue culture dishes at a density of 1×10^5 cells/cm^2. After 1–2 days, the monolayers had reached a final density of $2–3 \times 10^5$ cells/cm^2. Doxorubicin was added directly to the culture medium from a concentrated (2 mg/ml) aqueous stock solution which had been aliquoted and stored at −20 °C. After incubation for 3 h at 37 °C, cell monolayers were rinsed with phosphate-buffered saline (pH 7.4) to remove extracellular drug and irradiated at ambient temperature (about 23 °C) in phosphate-buffered saline containing 1 g/l glucose. Parallel control cultures were incubated under corresponding conditions in phosphate-buffered saline containing 1 g/l glucose. All cultures were trypsinized and plated for determination of clonogenic survival as previously described [7]. The figures show representative results of experiments that were performed from two to four times with similar results. The standard error of the clonogenic survival assays was routinely ±20%.

Laser light source

These studies employed an INNOVA 306 argon-ion laser (Coherent Laser Group, Palo Alto, Calif.) operating in the multiline mode. The 6 W power output was distributed mainly at 514.1 nm (40%) and 488 nm (30%), with the remainder being distributed at nearby wavelengths. The 3-mm beam was spread to either 2 cm^2 or 10 cm^2 depending on the size of the culture dishes being irradiated. Energy (fluence) delivered to the cultures was calculated by dividing the total energy output by the area of the spread beam and multiplying by the duration of exposure. Thus, 6 W delivered over a 2 cm^2 area for 300 s resulted in a fluence of 900 J/cm^2. Since previous data [9] had shown that cytotoxicity enhancement depends on fluence and not on fluence rate, the duration of exposure was varied to obtain the desired fluence.

Anoxic conditions

After incubation for 2 h with doxorubicin at 37 °C, culture dishes were transferred immediately to a transparent polystyrene chamber in a glove Atmosbag (CBS, Scientific, Delmar, Calif.). Both the Atmosbag and the chamber were connected to a cylinder delivering 99.98% pure N_2 gas (containing 0.02% CO_2 and <1 ppm O_2). Positive pressure with continuous nitrogen purging was maintained for 30 min prior to irradiation. The chamber was then sealed, removed from the Atmosbag and the N_2 flow maintained during light irradiation. The duration of exposure was increased 15% relative to that delivered to controls irradiated outside the box in order to compensate for attenuation of the beam by passage through the box wall.

DNA content analysis

Cell monolayers that had been plated on glass slides immersed in Petri dishes containing tissue culture medium were rinsed and irradiated as above. The cells were then fixed with 10% buffered formalin and stained according to CAS recommendations (B-D Cell Analysis Systems, Elmhurst, Ill.). The modified Feulgen method employed in this procedure yields nuclear stain optical densities that are proportional to DNA content. Analyses were performed using a CAS 200 instrument and Quantitative DNA Analysis version 3.0 software which relates the integrated optical density of specimen nuclei to that of standard calibration nuclei of known DNA content. All analyses were performed by the same operator to eliminate interobserver variation [3].

Results

Figure 1 shows that the sensitivity of the various lines to doxorubicin cytotoxicity differed greatly. While the L929 line was approximately as sensitive as the stock cells previously examined [9], the MCF-7 and RT-4 lines were nearly as resistant as the L929/DOX resistant clonal derivative. Nevertheless, clonogenic survival of all of the lines was decreased by light exposure following a period of doxorubicin uptake. The lines differed in dose-response details (as is readily apparent from the figure), so that the cytotoxicity of the intracellular drug was increased from 10-fold to 1000-fold depending on the line, the doxorubicin concentration, and the light dose delivered.

Figure 2 illustrates the nearly absolute dependence of light-enhanced cytotoxicity on molecular oxygen. It should be noted that this deceptively simple experiment required extensive optimization of nitrogen purging since shorter periods were not fully effective in removing dissolved oxygen from the medium and longer periods inhibited clonogenic survival. Anaerobiosis blocked light-enhanced cytotoxicity even though cultures were reexposed to atmospheric oxygen immediately following irradiation. Therefore, the results also support the conclusion that the oxygen-dependent step leading to enhanced cytotoxicity occurred during or very shortly after irradiation.

Figure 3 shows that within 24 h of treatment with doxorubicin alone (DOX), the L929 cell DNA content increased approximately twofold, i.e. from the "normal" amount (13 pg) to the 2*N* amount (26 pg). This finding is consistent with our previous observations on this cell line [8] following DOX exposure. In contrast, DNA loss was detectable within 4 h of light irradiation of doxorubicin-containing cells and was very pronounced within 24 h of irradiation. Virtually identical results were obtained after exposure to 0.3 μg/ml DOX, a drug concentration that results in relatively low cytotoxicity in the absence of irradiation. These results parallel qualitative observations of the clonogenic survival system where cells in irradiated doxorubicin-treated cultures are dead and shrunken while those treated with doxorubicin alone enlarge to form bizarre giant cells.

Discussion

In an earlier study [9], we demonstrated that the ability of argon ion laser irradiation to increase the cytotoxicity of intracellular doxorubicin is dependant on drug

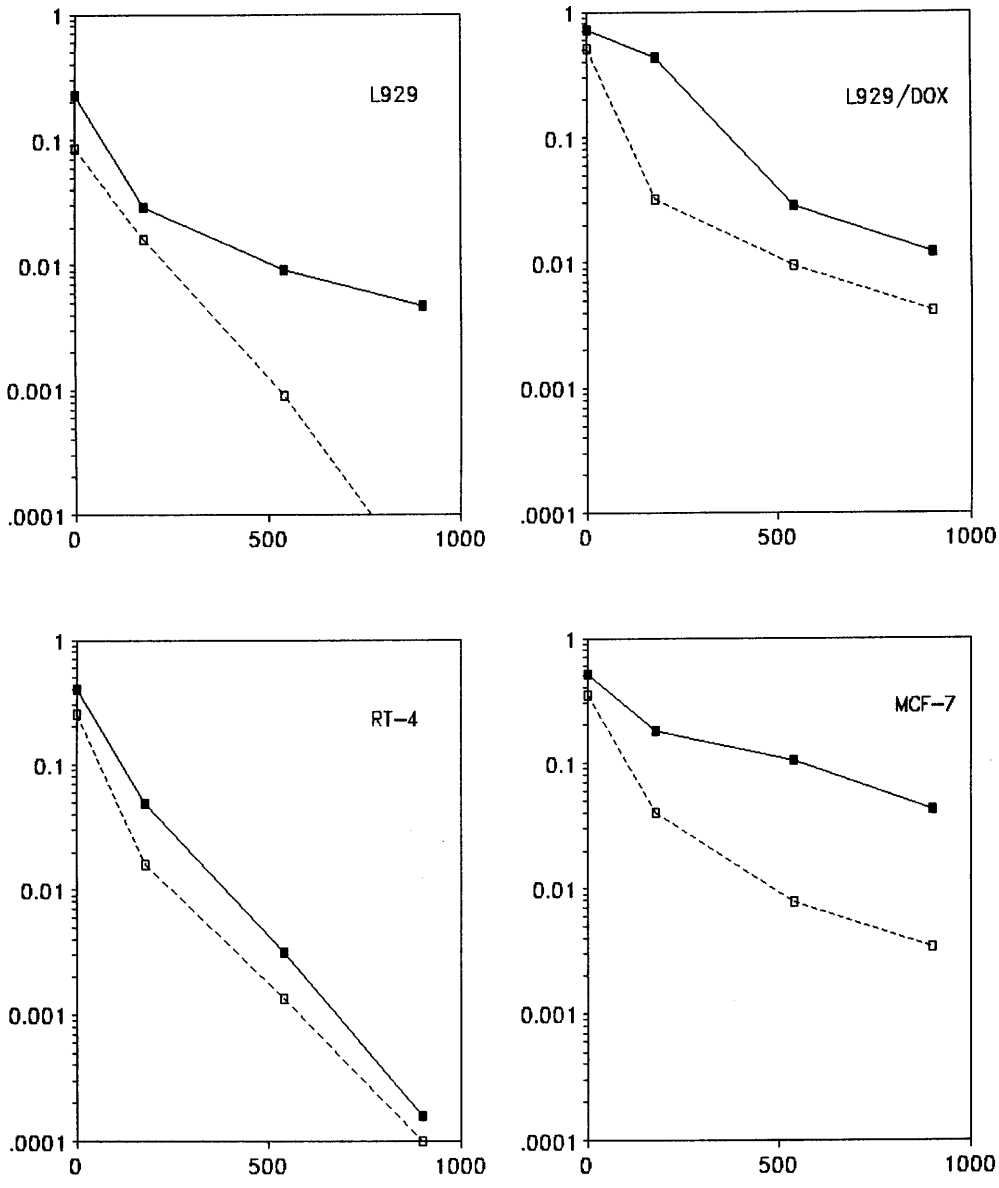
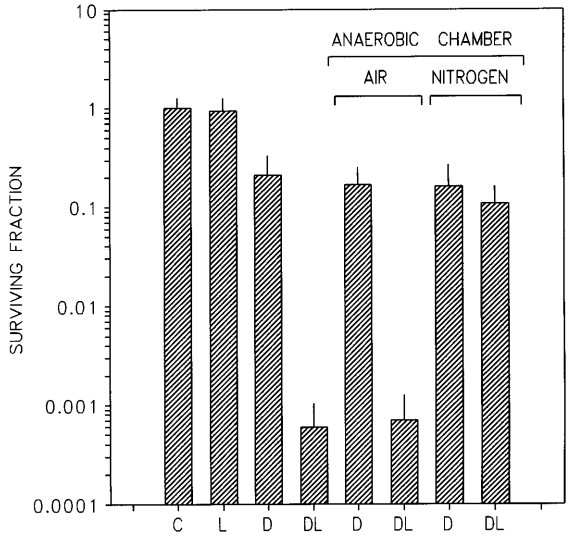


Fig. 1 Clonogenic survival of various cell lines (*ordinate*) after 3 h exposure to 3 μg/ml (■) or 5 μg/ml (□) doxorubicin followed by light exposure at the power density (J/cm^2) indicated on the *abscissa*



concentration and total light energy delivered. Inhibition of laser-enhanced cytotoxicity by catalase and superoxide dismutase suggests that the cytotoxic mechanism is dependent on the generation of active oxygen species by the photodynamically excited drug. In the present investigation, we extended these observations to additional cell lines, including a doxorubicin-resistant L929 derivative, and examined the mechanism of laser-enhanced cytotoxicity in more detail.

Drug resistance, either *ab initio* or emerging in the course of treatment, is a clinically important limitation

Fig. 2 Oxygen dependence of laser-enhanced doxorubicin cytotoxicity. L929 cell cultures were exposed to 3 μg/ml doxorubicin for 3 h and then irradiated with a fluence of 360 J/cm^2 as described in Materials and methods (*C* untreated control, *L* light alone, *D* doxorubicin alone, *DL* doxorubicin followed by light)

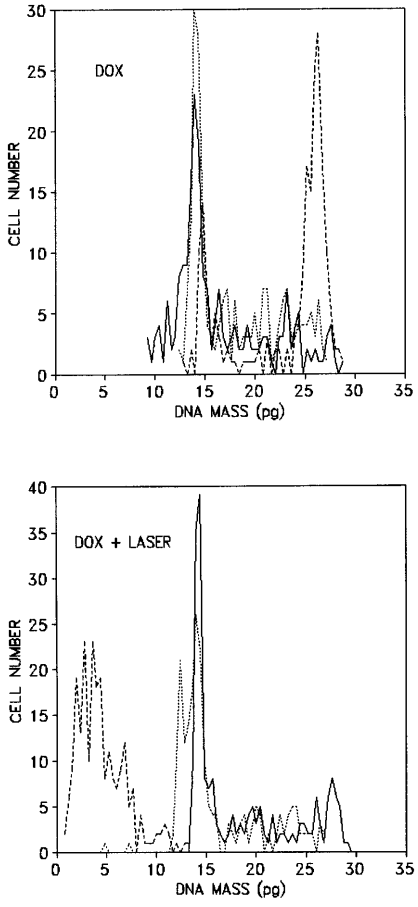


Fig. 3 DNA content of L929 cells exposed to 5 μg/ml doxorubicin for 3 h (*DOX*), or exposed to doxorubicin followed by 900 J/cm^2 argon ion laser irradiation, (*DOX + LASER*). Analyses were performed 0 h (——), 4 h (- - -), and 24 h (– – –) following treatment

of doxorubicin chemotherapy. In the present study, the breast and bladder carcinoma lines were relatively resistant without selection while the L929/DOX line modeled the emergence of resistance during treatment. Several mechanisms of doxorubicin resistance have been identified, including increased rate of drug extrusion [10] and increased activity of free radical detoxifying pathways [6, 13, 14, 16]. Although the mechanisms responsible for resistance of the various lines used in the present study were not determined, it is noteworthy that none of the lines was resistant to laser-enhanced cytotoxicity.

The results of both our previous studies showing inhibition by catalase and superoxide dismutase and the present experiments demonstrating inhibition by anoxia strongly suggest a mechanism of laser-enhanced doxorubicin cytotoxicity that is dependent on molecular oxygen. Numerous studies have attributed a component of doxorubicin cytotoxicity to the production of active oxygen species such as hydroxyl radical and superoxide [1, 13–15]. Since these mechanisms involve NADPH-dependent drug reduction [5, 12], laser irradiation may facilitate this process in some as-yet-unidentified way. However, the doxorubicin absorption spectrum also overlaps the argon ion laser emission spectrum to a considerable degree so direct electronic excitation of the drug followed by reaction with molecular oxygen also must be considered.

Experiments demonstrating extensive nuclear DNA loss from doxorubicin-treated laser-irradiated cells provide insights into the cytotoxic mechanisms of both conventionally administered and laser-irradiated doxorubicin. Despite the predominantly nuclear localization of the drug and production of single-strand breaks as a result of topoisomerase II inhibition [4, 11], treatment of L929 cells with doxorubicin alone is followed by progressively increasing DNA content as a result of continued DNA synthesis without cell division [8]. Even though a component of the drug's cytotoxicity can be attributed to NADPH-dependent free radical formation [5, 13, 16], this process does not appear to lead to wholesale DNA degradation. This result would be expected if NADPH-dependent free radical formation by doxorubicin were an entirely cytoplasmic process, so that the bulk of the intranuclear drug is unable to participate in this cytotoxic mechanism. In contrast, laser irradiation, which also appears to generate toxic free radicals, results in such extensive DNA damage because the reactive oxygen species are being produced in the nucleus where the bulk of the free drug is located.

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