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ORIGINAL ARTICLE

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Photodynamic enhancement of doxorubicin cytotoxicity

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Abstract Argon ion laser irradiation at 514.1 nm and 488 nm dramatically increased doxorubicin cytotoxicity in an L929 cell clonogenic survival assay. The cytotoxicity was dependent on both the drug concentration and the total light energy delivered such that at 5 μg doxorubicin/ml and 800 J/cm^2, cytotoxicity was enhanced by a factor of $>10^4$ relative to that achieved with drug alone. Irradiation times in excess of 2 min and power densities in excess of 100 J/cm^2 were required to produce the effect. Beyond this 2-min limit, cytotoxicity was not related to the duration of exposure if the total energy delivered was held constant. The ability of catalase and superoxide dismutase to abolish completely the increase in cytotoxicity produced by laser irradiation suggests that the cytotoxic mechanism may depend on the generation of active oxygen species by the photodynamically excited drug.

Key words Doxorubicin · Adriamycin · Photochemotherapy · Cancer chemotherapy

Introduction

The ideal cancer chemotherapeutic agent is both highly cytotoxic and specific for neoplastic cells. Photochemotherapy approaches this goal by using directed beams of light in combination with systemic administration of chemical agents to produce cytotoxic photoactivation products such as singlet oxygen in the immediate vicinity of cancer cells. Although initially employing hematoporphyrin derivatives [7], recent efforts in this field have been directed toward developing a wider variety of drugs that are susceptible to photoactivation.

One of the recognized cytotoxic mechanisms of doxorubicin, a widely used cancer chemotherapeutic agent, depends on drug bioreduction followed by reaction with molecular oxygen and generation of reactive oxygen species [2, 10, 11]. Since doxorubicin has an absorption maximum at 579 nm and since electronically excited molecules may be highly reactive, photoactivation of this drug might lead to enhanced cytotoxicity by initiating a mechanism similar to that initiated by bioreduction. Even though previous attempts to enhance cytotoxicity by irradiation using conventional light sources have failed [1], we pursued this goal with considerable success using a laser source matched to the absorption spectrum of the drug.

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Materials and methods

Reagents and chemicals

Doxorubicin was obtained from Farmitalia Carlo Erba (Milan, Italy). Catalase and superoxide dismutase were supplied by Sigma (St. Louis, Mo.).

Cells and culture conditions

Murine L929 cells (a permanent fibroblast-related line) 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 as previously described [9]. Cells from the stock cultures were plated in 35-mm plastic tissue culture dishes or in multiwell plates 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) stock solution. After incubation for 2 h at 37 °C, cell monolayers were rinsed with phosphate-buffered saline (pH 7.4) to remove extracellular drug and irradiated at ambient temperature (ca. 23 °C) in serum-free DMEM lacking phenol red. In experiments employing superoxide dismutase or catalase, enzymes (10 μg/ml) were added following doxorubicin exposure and immediately before laser irradiation. Parallel control cultures were incubated in phosphate-buffered saline under corresponding conditions. All cultures were trypsinized and plated for determination of clonogenic survival as previously described [9]. Although Figs. 1–7 show representative data, all experiments 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 power output was distributed mainly at 514.1 (40%) and 488 nm (30%), with the remainder being distributed at nearby wavelengths. The 3-mm beam was spread to either 2 or 10 cm^2, depending on the size of the culture dishes being irradiated. Energy delivered to the cultures was calculated by dividing the total energy output by the area of the spread beam. The temperature of the culture medium before and immediately following irradiation was measured using an IBM PSL probe with a precision of ± 0.01 °C. Physiological effects of heating that occurred during irradiation were assessed by labeling with [^{35}S]-methionine, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography as previously described [8] for detection of heat-shock protein synthesis.

Results

Irradiation for 5 min at 3 W/cm^2 after exposure to doxorubicin at 5 μg/ml decreased clonogenic survival by a factor of $>10^4$ as compared with cultures exposed to the drug alone (Fig. 1). Irradiating these cells at 0.1 μg doxorubicin/ml for 5 min at the maximally attainable power (3 W/cm^2) had little effect on clonogenic survival (Fig. 1). Irradiation for 5 min at 3 W/cm^2 in 2-cm^2 culture wells raised the temperature of the medium from 22.6° to 30.0 °C, i.e., an increase of 7.4 °C. Spreading the beam to 10 cm^2 and irradiating 10-cm^2 dishes for 5 min raised the temperature by 4.1 °C. Irradiating 10-cm^2 dishes for even 20 min did not cause physiologically significant heating of the systems since heat shock protein synthesis was not induced (data not shown).

Laser irradiation increased cytotoxicity at all drug concentrations examined, even in the range of 0.1–0.5 μg/ml, where no significant cytotoxicity was apparent in nonirradiated cultures. The cytotoxicity observed at 0.1 μg/ml could be attributed to the additive effects of drug plus irradiation (Fig. 2).

In contrast, Fig. 3 shows that irradiation for >2 min (following exposure to doxorubicin at 0.5 μg/ml for 2 h)

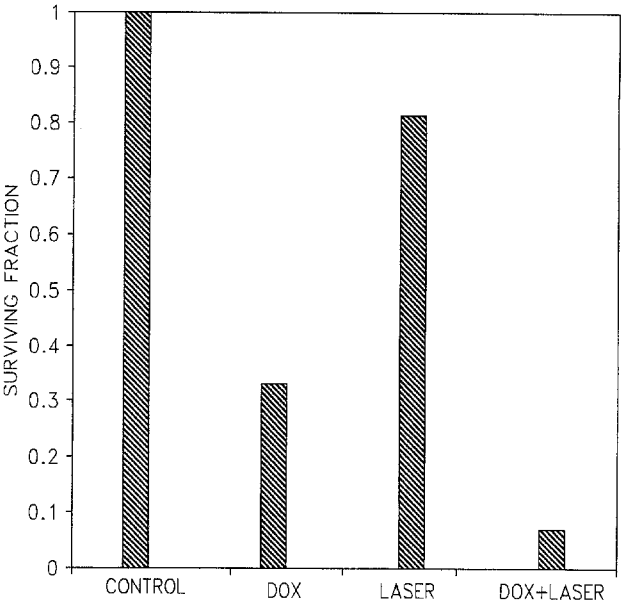


Fig. 2 L929 cell clonogenic survival following 2 h exposure to doxorubicin alone (*DOX*, 0.1 μg/ml), 3 W/cm^2 argon-ion laser irradiation for 5 min (*LASER*), and irradiation after 2 h doxorubicin uptake (*DOX+LASER*). Data points represent the mean cloning efficiencies of duplicate drug-treated cultures relative to the corresponding controls (SD, ≈20%)

Fig. 1 L929 cell clonogenic survival after 2 h exposure to doxorubicin alone (■—■) or to doxorubicin followed by 3 W/cm^2 argon ion laser irradiation for 5 min (□ --- □). Data points represent the mean cloning efficiencies of duplicate drug-treated cultures relative to the corresponding controls (SD, ≈20%)

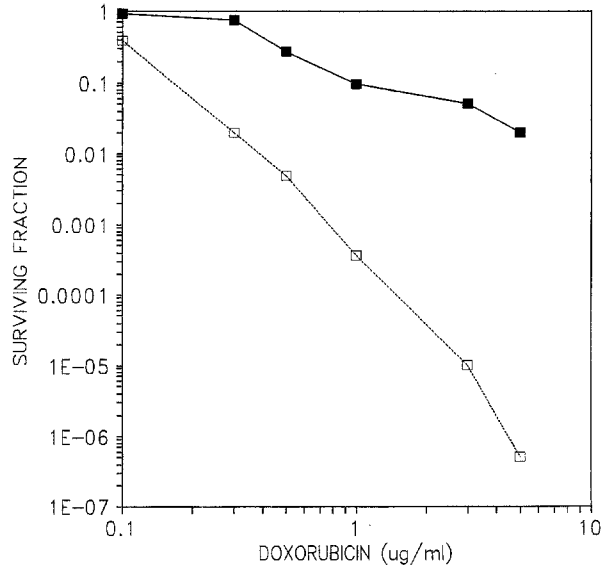
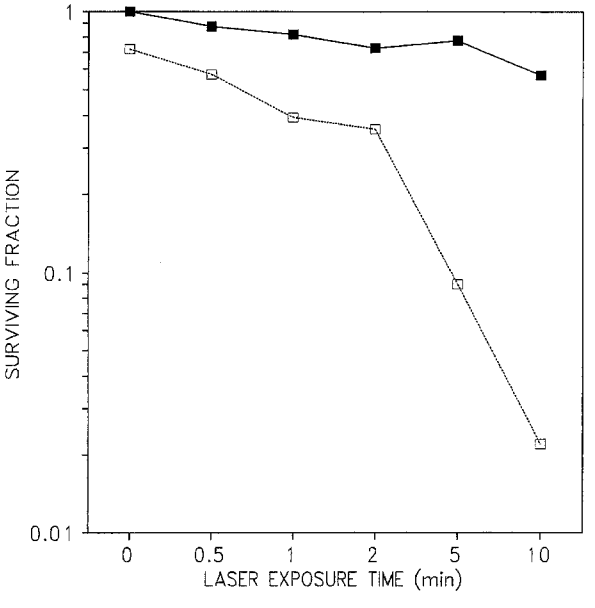


Fig. 3 Dependence of L929 cell clonogenic survival on laser exposure time (3 W/cm^2). Cultures were irradiated for the indicated times without drug exposure (■—■) or following 2 h exposure to doxorubicin at 0.5 μg/ml (□ ---□). Data points represent the mean cloning efficiencies of duplicate drug-treated cultures relative to the corresponding controls (SD, ≈20%)



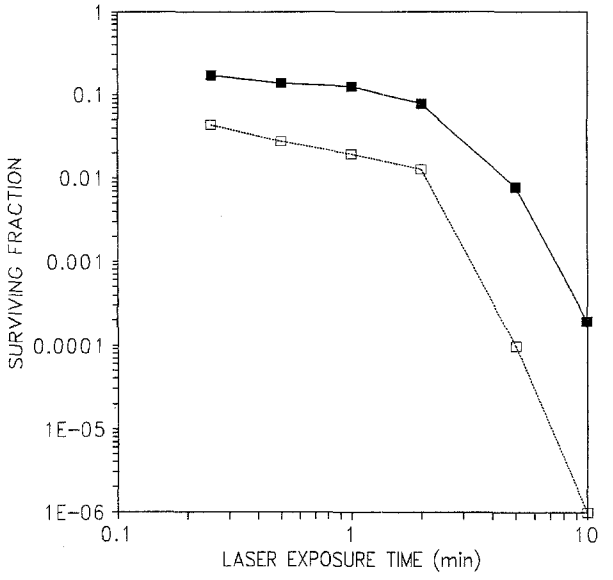


Fig. 4 Dependence of L929 cell clonogenic survival on laser exposure time. Cultures were irradiated (3 W/cm²) for the indicated times following exposure to doxorubicin at 3 (■———■) or 5 μg/ml (□---□). Data points represent the mean cloning efficiencies of duplicate drug-treated cultures relative to the corresponding controls (SD, ≈20%)

synergistically enhanced cytotoxicity. This interpretation of the data is based on the relatively minor decrease in clonogenic survival observed with irradiation alone for >2 min as compared with the relatively large decrease in clonogenic survival observed when similar irradiation was carried out following doxorubicin exposure. Figure 4 shows that the synergistic enhancement of cytotoxicity at relatively high drug concentrations also required a duration of irradiation in excess of 2 min. Beyond this 2-min limit, cytotoxicity was not related to the duration of exposure if the total energy delivered was held constant (Fig. 5). These observations suggest that it might be necessary to exceed a threshold power density before cytotoxicity is enhanced.

Since the duration of irradiation was held constant during the experiments illustrated in Fig. 5, additional experiments were performed to determine whether the apparent 100-J/cm² threshold truly reflected a minimal total-energy requirement. This consideration was felt to be important because clinical applications might preclude irradiation at the intensities achieved in vitro. Figure 6 confirms that power densities in excess of 100 J/cm² were required to produce this effect.

The question as to whether laser irradiation enhanced doxorubicin cytotoxicity by the generation of active oxygen species was approached by examining the ability of catalase and superoxide dismutase to block the effect. Figure 7 shows that either enzyme was capable of completely abolishing the increase in cytotoxicity produced by laser irradiation, i.e., exposure of cultures to doxorubicin followed by enzyme was the same in the presence or absence of irradiation. It should be noted that enzyme treatment did not completely block doxorubicin cytotoxicity since drug and enzyme were not applied simultaneously [3].

Discussion

Photochemotherapy employs visible (or near-infrared) light in conjunction with a photosensitizing compound for selective killing of tumor cells [7]. Most current work

Fig. 5 Dependence of L929 cell clonogenic survival on laser power density. Cultures were irradiated for 5 min at the indicated power densities following exposure to doxorubicin at 5 μg/ml in either 10-cm² dishes (■—■) or 2-cm² culture wells (□---□). Data points represent the mean cloning efficiencies of duplicate drug-treated cultures relative to the corresponding controls (SD, ≈20%)

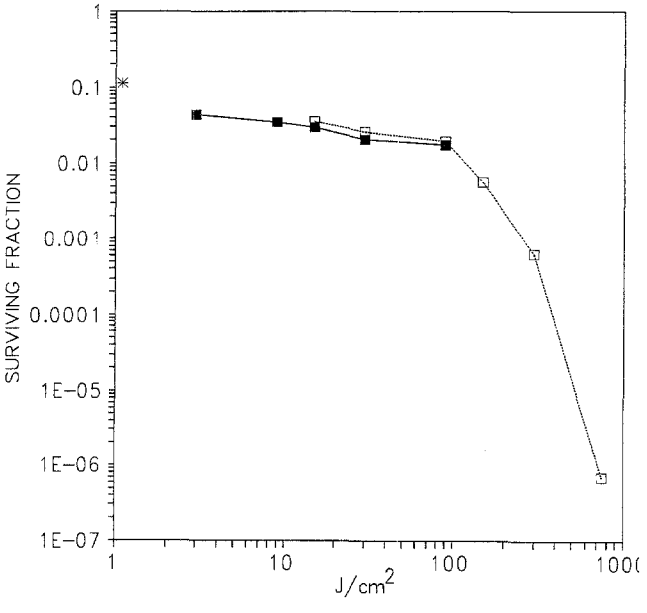
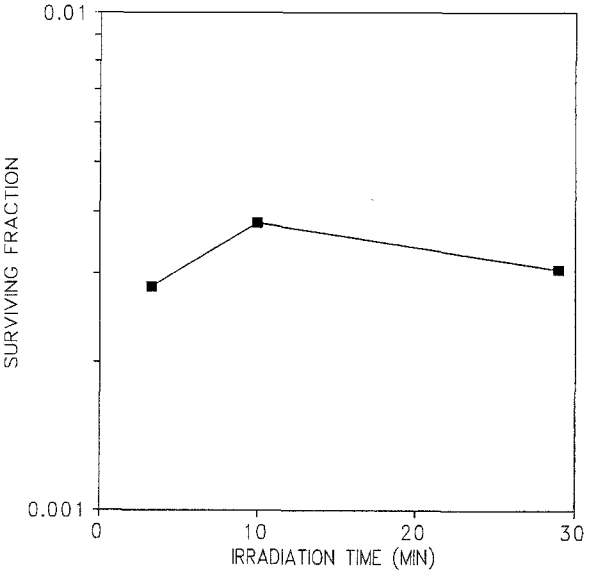


Fig. 6 Dependence of L929 cell clonogenic survival on laser power. Cultures were exposed to doxorubicin at 3 μg/ml and then irradiated at light-source intensities ranging from 0.67 to 6 W/cm². The duration of exposure was adjusted to yield 600 J/cm² total energy. Data points represent the mean cloning efficiencies of duplicate drug-treated and irradiated cultures relative to the untreated controls (SD, ≈20%)



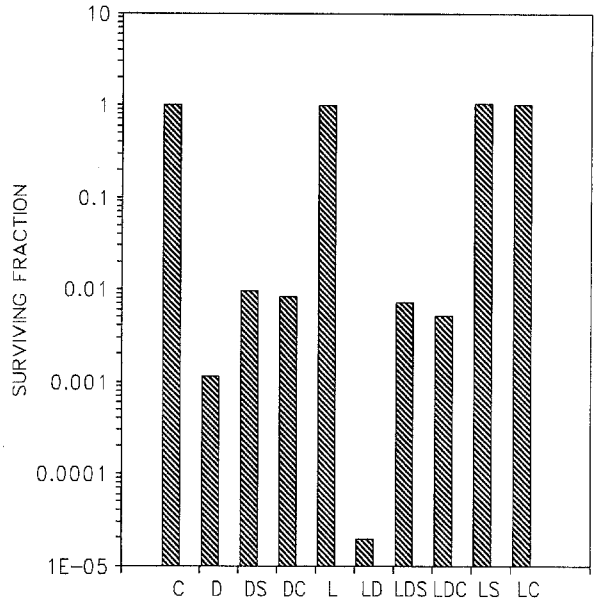


Fig. 7 Inhibition of doxorubicin photodynamic activation by catalase or superoxide dismutase. Cultures were exposed to combinations of agents as follows: *D*, doxorubicin (5 μg/ml) for 2 h; *L*, 1 W/cm^2 laser irradiation; *C*, *S*, catalase (10 μg/ml) or superoxide dismutase (10 μg/ml), respectively, following doxorubicin exposure and during laser irradiation

employs porphyrin derivatives that are selectively taken up by tumor cells, where they are essentially nontoxic unless exposed to light. Since clinical applications remain limited by the availability of appropriate sensitizing drugs, many groups, e.g., al-Nabulsi and Gutierrez [10], are attempting to develop agents that are chemically and mechanistically different from porphyrins. To this end we examined the photosensitizing potential of doxorubicin, a commonly used cancer chemotherapeutic agent that absorbs strongly in the visible spectrum.

Argon-ion laser irradiation at wavelengths near the absorption maximum of doxorubicin dramatically increased the cytotoxicity of this drug in the L929 clonogenic survival assay system. Although predicted on the basis of theoretical considerations, this effect has been impossible to achieve by other approaches [1]. Elevated temperature is known to increase doxorubicin cytotoxicity [5], but heating does not appear to be responsible for the increased cytotoxicity that we observed. This is so because temperatures necessary to induce heat-shock protein synthesis are required and this response was not observed. In fact, cultures were never exposed to supraphysiological temperatures during the course of these experiments.

The cytotoxic mechanism appears to depend on the generation of active oxygen species by the photodynamically excited drug. Although photodynamically excited porphyrins are cytotoxic by virtue of singlet oxygen production [7], our observations that catalase and superoxide dismutase block photodynamically enhanced doxorubicin cytotoxicity implicate peroxide and superoxide in the present mechanism. These species are also important in the conventional mechanism of doxorubicin-mediated cell killing [11]. This finding supports a reaction sequence similar to that initiated by drug bioreduction in which the semiquinone product reacts with molecular oxygen to produce superoxide.

The ability to increase doxorubicin cytotoxicity by photoactivation may have applications in cancer chemotherapy. It is well known that extracellular glucose concentrations are very low in some tumor regions [4]. Since reduced nicotinamide adenine dinucleotide phosphate (NADPH) synthesized by metabolizing glucose is an essential component in one of the doxorubicin cytotoxic mechanisms, a lack of glucose may protect some tumor cells against this drug. Such cells cannot also be anoxic because deprivation of both oxygen and glucose is not compatible with survival [6]. Therefore, direct generation of active oxygen species by photoactivation should be capable of eliminating any cells that are resistant to doxorubicin by virtue of glucose deprivation.

The observation that enhancement of cytotoxicity is related to the total energy delivered at irradiation times in excess of 2 min also has therapeutic implications. Irradiating large areas of tissue at the intensities achieved in vitro may be impractical because of hardware limitations and tissue heating. However, our data suggest that this problem can be circumvented simply by extending the duration of exposure.

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