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Cytotoxic effects and pharmacokinetic analysis of combined adriamycin and X-ray treatments in human organotypic cell cultures

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Organotypic cultures of human A549 cells were used as a tumor model to investigate sequence effects for combination treatments with adriamycin (ADR) and X-irradiation. Initial drug exposure led to the greatest cytotoxic effect especially when X-rays were delivered 24 h later and this subsequent irradiation did not significantly modify the intracellular ADR concentration. In contrast, post-irradiation drug exposure gave rise to a lower cytotoxic effect, and induced a marked reduction of intracellular and more specifically intranuclear ADR uptake and retention, especially when the drug was given 24 h later.

Key words: A549 cell line, adriamycin, X-rays.

Introduction

Adriamycin (ADR), an anthracycline widely used in cancer chemotherapy,[1] has been recognized for a long time to be a potential radiosensitizer both *in vitro* and *in vivo*.[2,3] The synergistic cytotoxic efficacy of various combinations of ADR with X-rays has been ascribed to: (1) slowed proliferative rate, which occurs after irradiation;[4] (2) inhibition of oxygen consumption which may consequently favor reoxygenation of the radioresistant hypoxic cells;[5,6] (3) decreased capacity of cells to accumulate sublethal damage with no concomitant effect on the intrinsic radiosensitivity;[7,8] and (4) inhibition of repair of potentially lethal damage.[9] In addition, other parameters,[7,8] mainly the dose of ADR[10] and the time sequence between ADR and radiation applications,[11,12] have been shown to influence the effectiveness of these combined effects. However, no data concerning the modifications of ADR pharmacokinetics during combined treatments have yet been reported. We therefore conducted experiments designed to examine the nature of ADR and X-ray interactions as a function of drug concentration and of time sequence between the two treatments using an *in vitro* three-dimensional model (organotypic cultures) of A549 cells. The parameters analyzed here were the cytotoxicity of combined ADR and X-irradiation on organotypic cultures of A549 in relation to ADR pharmacokinetics, i.e. uptake, retention and intracellular partition of the drug analyzed by high pressure liquid chromatography (HPLC).

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Material and methods

Organotypic cultures

In vitro organotypic cultures (nodules) of A549 cells derived from a human lung adenocarcinoma[13] were performed as previously described.[14–17] Briefly, a monolayer of A549 cells was scrapped off from a plastic flask (Falcon), and cells were aggregated by centrifugation (2000 r.p.m., 20 min). The cell pellet was placed on a semi-solid culture medium obtained by mixing at 1:1 ratio 1% Agar (Difco, Detroit) in distilled water and 2 × RPMI-1640 medium supplemented with 20% fetal calf serum in a petri dish and tightly closed. Nodules were subcultured every

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10 days by cutting them into two or four pieces with microsurgical scissors and were routinely checked for mycoplasma contamination (BRL mycotest).

ADR and X-ray treatment schedules

Nodules of A549 cells were kept in contact for 1 h with ADR (0.01 and 0.1 μg/ml) as previously described.[16,17] X-irradiation was performed at room temperature with an X-ray machine (Vega) at 250 kV and 11 mA with a 2 mm aluminum filter. The dose rate was 1 Gy min and the total dose delivered was 5 Gy. Treatment schedules were as follows: (1) ADR given immediately after X-irradiation (X.ADR) or following a 24 h delay (X-ADR) and (2) X-irradiation given immediately after but in the presence of ADR (ADR.X) or following a 24 h delay (ADR-X).

Cytotoxicity

Measurements of nodule growth were carried out every 5 days. The rate of nodule growth was calculated as described previously;[15,16] it was expressed as a percentage, compared with that of untreated (control) nodules. Experiments were performed in triplicate and each value represents the average from 16 nodules. The significance of the data obtained was checked by Student's *t*-test.

HPLC analysis

ADR extraction was performed according to Robert's method.[18] Intracellular and intranuclear ADR measurements were performed by HPLC according to a previously described protocol.[16] Briefly, the analysis was performed using a Waters Associates liquid chromatograph with a μ-Bondapack C-18 column and precolumn. The solvent was used isocratically at a flow rate of 2 ml/min and drug fluorescence was identified by spectrofluorometry (Kontron); the excitation and emission wavelengths being 480 and 592 nm, respectively. Each sample was analyzed for ADR content in triplicate from 60 nodules and the error bar represents the standard deviation of three separate samples for which the intracellular ADR concentration was normalized to the DNA content.[16,17]

Results

The cytotoxicity of ADR application, X-irradiation, and combined ADR and X treatments was assessed by measuring the extent of nodule growth every 5 days. The extent of nodule growth at day 10 following each initial treatment was normalized to that of untreated control nodules (growth of control nodules at day 10 was 120%). The values obtained at ADR concentrations of 0.1 μg/ml and 0.01 μg/ml are represented in Figure 1 showing that the extent of nodule growth of ADR (0.1 μg/ml) or X-ray treated nodules was 60 and 71%, respectively. The combination of X-rays and ADR did not give rise to an additive effect especially when ADR was given after X-irradiation (X-ADR) (68%). In contrast, the combined treatments induced a marked cytotoxic effect when ADR was given 24 h before X-irradiation (ADR-X; 22%). These results, also observed for an ADR concentration of 0.01 μg/ml (Figure 1), prompted us to measure ADR pharmacokinetics by HPLC in order to study the uptake, retention and intracellular partition of the drug following each treatment schedule.

Figure 2 shows that, following an ADR treatment at 0.1 μg/ml, the intracellular ADR concentration of 3.4 ng/μg DNA obtained 1 h after application (uptake) remained stable 24 h later in the absence of the drug (retention). These values were not significantly modified when X-rays were delivered directly or 24 h after ADR application (ADR.X and ADR-X, respectively). Moreover, in the case of ADR-X treatment a further dosage was

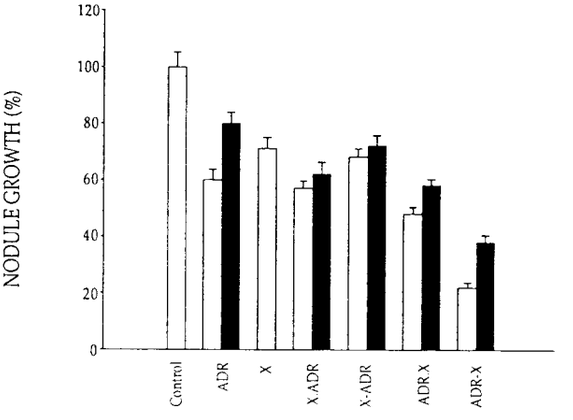

Figure 1. Extent of nodule growth (%) at day 10 normalized to that of untreated control nodules (100%) following each initial treatment. ADR concentrations are 0.1 (□) and 0.01 μg/ml (■).

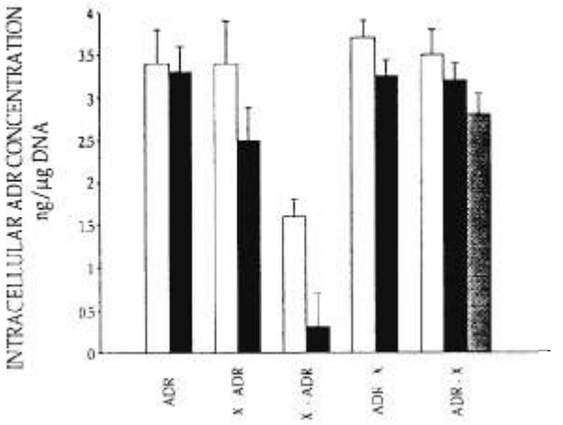

Figure 2. Intracellular ADR concentrations (ng/μg DNA) measured 1 (□), 24 (■) and 48 h (▨) after each initial treatment at ADR concentration of 0.1 μg/ml.

performed 48 h after ADR application in order to be able to always compare drug retention 24 h after X-irradiation. The data obtained did not show any significant modification as compared with that of a single ADR treatment (Figure 2). Intracellular ADR concentrations were also measured when X-rays were delivered directly or 24 h before ADR application (X.ADR and X-ADR, respectively). Data presented in Figure 2 show that X.ADR treatment induced a slight reduction of drug retention without any modification in drug uptake, whereas X-ADR treatment induced a marked decrease in both intracellular ADR uptake and retention.

In addition, following each treatment schedule assays of intranuclear ADR content were carried out and the values obtained were normalized to the respective intracellular ADR concentrations measured. Table 1 summarizes the calculated data, expressed as a percentage, showing that intracellular ADR partition was not modified when X-rays were delivered after ADR application, whereas a significant reduction of intranuclear ADR concentration was observed without any modification of intranuclear drug uptake following X.ADR treatment. In contrast, a marked reduction of both intranuclear ADR uptake and retention was noted following X-ADR treatment, clearly indicating that primary X-irradiation interferes with the intracellular ADR partition by inducing transfer of the drug from the nucleus to the cytoplasm, particularly when X-rays were delivered 24 h before ADR application.

Discussion

Three-dimensional organotypic cultures (nodules) of human A549 cells have been shown to be an attractive model for analyzing the efficiency of combined treatments using various drugs and X-irradiation.[14–17,19] The present work reports results of a study designed to test the cytotoxicity of different treatment schedules associating ADR and X-rays, and to investigate by means of HPLC analysis the uptake, retention and intracellular partition of ADR using drug concentrations similar to those currently encountered in clinical trials.

Our results show the striking effect of the ADR-X association, i.e. the effectiveness of primary ADR application followed by X-irradiation 24 h later. Similar results have been reported in various *in vitro* models by other investigators. Belli *et al.*[12] showed that ADR pretreatment reduced the shoulder of radiation survival curves, and that this reduction was observed for up to 24 h after ADR application. In contrast, primary X-irradiation did not give rise to a similar phenomenon. These authors also emphasized the critical effect of ADR concentrations, since additive and synergistic responses were observed for high and low levels of ADR, respectively. Similarly, Durand *et al.*[5,6] using another three-dimensional system termed spheroids showed a supra-additive response when high doses of ADR were given before X-rays and ascribed this effect to a drug-induced spheroid reoxygenation. Such a mechanism can be excluded in our model since no hypoxic gradient was observed inside the nodules.[14] Finally, Byfield *et al.*[10] described a synergistic response using primary ADR treatment even when X-rays were delivered 6 days after ADR

Table 1. Intranuclear ADR content (% ±SD) normalized to the respective intracellular ADR concentrations measured before (t 0), and 24 (t 24) and 48 h (t 48) after each initial treatment at ADR concentration of 0.1 μg/ml

ADR		X.ADR		X-ADR		ADR.X		ADR-X		
t 0	t 24	t 0	t 24	t 0	t 24	t 0	t 24	t 0	t 24	t 48
92 ± 6	88 ± 10	89 ± 4	62 ± 6	47 ± 3	10 ± 1	91 ± 9	98 ± 7	89 ± 6	95 ± 4	87 ± 5

application and postulated that this effect could be attributed to the presence of a limited fraction of ADR remaining intercalated into nuclear DNA.

In this regard, our results clearly establish that intracellular ADR retention is significantly modified by primary X-irradiation. Moreover, the unaffected intranuclear ADR concentrations observed following this treatment schedule reinforce our reported data concerning cytotoxicity, bearing in mind that the main target of ADR is DNA. In contrast, a reverse treatment schedule, namely X-irradiation delivered 24 h before ADR application, gave rise to a marked reduction of both intracellular and intranuclear ADR uptake and retention. Such a phenomenon could be explained by various hypotheses. X-rays may induce a drop in intranuclear pH which is known to lead to a redistribution of the drug[20–22] and to prevent the DNA intercalation of ADR, and/or it may favor the efflux of the drug by chemically modifying the nuclear and cytoplasmic membranes[23]. Alternatively, as already described following γ-irradiation,[24] it could be hypothesized that primary X-irradiation furthers the enzymatic degradation of ADR into drug metabolites which are known to be easily expelled from the cells and to be less cytotoxic than ADR.[25–27] This detoxification pathway which has been shown to actually take place in our model,[16,17] could explain the present reported pharmacokinetic and cytotoxic results. We were, however, unable to investigate this hypothesis since the ADR concentrations used in our study were deliberately chosen in the range of those currently employed in clinical trials, thus preventing the measurement of minute amounts of ADR metabolites by means of HPLC analysis.

Conclusion

Pre-irradiation gives rise to a significant reduction of subsequent intranuclear ADR uptake and retention in these organotypic cultures of human A549 cells, especially when ADR is given following a 24 h delay. In contrast post-irradiation does not modify the intracellular concentration of ADR and results in the greatest cytotoxic effect, especially when X-rays are delivered 24 h after the drug.

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