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ORIGINAL ARTICLE

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Interactions of mild hyperthermia, cisplatin and split dose irradiation in human ovarian carcinoma cells

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Abstract *Purpose*: Human ovarian cancer cells were evaluated to determine whether combination treatment with mild hyperthermia and cisplatin could inhibit repair of sublethal radiation damage. *Materials and methods*: Human ovarian carcinoma cell lines A2780S (parental line) and A2780CP (cisplatin resistant variant) were used in this study. Cisplatin at concentrations of 1 or 3 μg/ml was given concomitantly with 1 h of heating at 40 °C either immediately before or after irradiation. Survival was determined using a colony-forming assay. *Results*: Neither mild hyperthermia nor cisplatin treatments alone affected sublethal damage repair. The combined treatment showed an effect in both cell lines and was treatment sequence-dependent. The effect was greater in the parental cell line. *Conclusions*: The data show that combined treatment of cisplatin and hyperthermia may have clinical efficacy at cisplatin concentrations and hyperthermia temperatures which by themselves have little to no effect.

Key words Hyperthermia · Cisplatin · Radiation · Ovarian carcinoma · Combined treatment

Introduction

The cellular response to radiation is influenced by the repair of radiation damage. Both sublethal damage repair (SLDR) and potentially lethal damage repair (PLDR) have been extensively studied (Elkind and Sutton 1960; Little 1969; Philips and Tolmach 1966; Whitmore and Culyas 1967). In our own studies we have shown that the extent of damage that cells repair may be much greater than that revealed by the conventional techniques used to study SLDR and PLDR. Using anisotonic treatment after irradiation, an extensive amount of damage is expressed, which would otherwise be repaired (Raaphorst and Dewey 1979a; Raaphorst and Dewey 1979b). Many studies have been directed at evaluating the inhibition of repair as a method for the enhancement of the effectiveness of radiation in order to improve radiotherapy. Both drugs and hyperthermia have been studied as repair inhibitors and it has been shown that repair inhibition can increase the effectiveness of radiation in cell killing (Berger et al. 1979; Nakatsugawa et al. 1984; Spadari et al. 1986; Sugahara et al. 1984; Ward 1986). We have shown that hyperthermia can inhibit both SLDR (Raaphorst 1992; Raaphorst et al. 1979; Raaphorst et al. 1994) and PLDR (Raaphorst and Feeley 1994; Raaphorst et al. 1993). Indeed, it has been shown that in tumor cells which are resistant to irradiation because of an increased repair capacity, the inhibition of such repair results in large increases in radiosensitivity (Raaphorst 1992; Raaphorst and Feeley 1994; Raaphorst et al. 1993).

Cisplatin is a well-known chemotherapy agent and it has also been used extensively in combined treatment with radiotherapy (Coughlin and Richmond 1985; Dewit et al. 1985; Kuske et al. 1989; Piver 1984; Sledge 1992; Taylor et al. 1994; Zietman et al. 1993) since it has been shown to be an effective radiosensitizer. In vitro studies using a wide variety of mammalian cells have shown that dose modifying factors (DMFs) of up to 1.4 can be achieved (Begg 1990; Begg et al. 1986; Dewit 1987; Dritschilo et al. 1979; Nias 1985; Walter et al. 1993; Wilkins et al. 1993). We have also shown in ovarian carcinoma cells that DMFs of up to 1.5 can be achieved and that the DMFs are larger in cisplatin-resistant cells than in the parental sensitive cell line (Raaphorst et al. 1995). This study showed the potential for therapeutic gain when combining cisplatin and irradiation in cells that are resistant to cisplatin treatment.

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Several studies have shown that radiosensitization by cisplatin may in part be through the inhibition of repair of radiation damage and both inhibition of SLDR and PLDR have been observed (Carde and Laval 1981; Douple 1990; Dritschilo et al. 1979; Schwachofer et al. 1991; Szumiel and Nias 1976; Wallner and Li 1987; Wilkins et al. 1993). We have shown in a rat 9L cell line, which is very radiation resistant, possibly in part through increased ability to repair radiation damage, that cisplatin can inhibit PLDR and that this inhibition is dependent on treatment sequence (Wilkins et al. 1993). Thus, cisplatin may be effective in the radiosensitization of cells which are radiation resistant on the basis of an elevated repair capacity. Indeed, Herman and Teicher (1988) and Herman et al. (1990) have shown that the use of cisplatin, hyperthermia and radiation together is effective, and this may be related to both hyperthermia and cisplatin showing a combined effect on repair of radiation damage.

A number of studies have shown that resistance to cisplatin treatment occurs in a wide range of mammalian cells. Several mechanisms of resistance have been identified and these include: reduced cisplatin uptake, increased levels of metallothioneins, increased levels of glutathione and increased repair capacity (Bosscha et al. 1993; Canon et al. 1990; Eastman and Schulte 1988; Fujii et al. 1994; Ireland et al. 1994; Jekunen et al. 1994; Masuda et al. 1988; Meijer et al. 1990; Micetich et al. 1983; Raaphorst et al. 1995). The last of these has been coupled with elevated levels of unscheduled DNA synthesis and increased activity of DNA repair enzymes (Eastman and Schulte 1988; Ireland et al. 1994; Masuda et al. 1988; Micetich et al. 1983; Raaphorst et al. 1995). Since elevations in repair capacity may affect radiation resistance, strategies that result in inhibition of repair of radiation damage may be effective, especially when used in combination with radiation in cells that are resistant through having elevated repair capacity.

Since both cisplatin and hyperthermia treatments have been shown to inhibit radiation damage recovery, we set out to test the effect of combined treatment in ovarian carcinoma cell lines sensitive and resistant to cisplatin and radiation. We chose a mild hyperthermia temperature which is easily clinically achievable and which by itself does not affect repair of radiation damage.

We chose to study cell cultures grown in the plateau phase in order to avoid cell cycle redistribution. In previous studies it has been shown that the cellular cisplatin response is affected by cell cycle phase (Krishnaswamy and Dewey 1993) and also by growth state (Raaphorst et al. 1996), and thus we attempted to hold these constant by having the cells in the plateau phase.

Materials and methods

The human ovarian carcinoma cell system used in this study was originally derived from an untreated patient and classified A2780S (Hamilton et al. 1989). The $A2780^{cp}$ is a cisplatin-resistant variant obtained by chronic exposure of A2780S to stepwise increasing concentrations of cisplatin up to 12 μg/ml (Hamilton et al. 1989).

Cells were grown in a 1:1 mixture of DMEM and F-12 medium, supplemented with 7.5% fetal bovine serum, 7.5% newborn calf serum, 0.1 m*M* MEM nonessential amino acids, 10 m*M* sodium bicarbonate, and 20 m*M* HEPES, and incubated at 37 °C in a humidified atmosphere of 2% CO_2 and 98% air. Experiments were done for cells grown into plateau phase at which point all cell cultures were confluent. For plateau phase, cells were fed with fresh medium 2 days before the experiment and the plating efficiencies ranged from 30% to 35% and from 50% to 55% for the A2780S and A2780CP lines, respectively.

Cisplatin was obtained as Cisplatin Injection (David Bull Canada, Vaudreuil, PQ) consisting of 1 mg/ml (3.33 m*M*) cisdiaminedichloroplatinum (II) and 9 mg/ml NaCl, pH adjusted to 7.3. Cells were treated by adding a measured amount of this solution directly to the culture medium covering the cells. At the end of the exposure period, the medium containing cisplatin was aspirated, and cells were rinsed twice with isotonic citrate saline and new medium was added. Cisplatin treatments were given for 1 h unless otherwise indicated in the Results section. For the sequential treatments, the cisplatin exposure was either terminated 5 min before or started 5 min after the radiation treatment.

For the SLDR assay, the cells were irradiated with a 4-Gy dose of X-rays and then incubated at 37 °C for the times indicated in the results section and then given a second radiation dose of 4 Gy. Cisplatin treatment was given either for 1 h before or for 1 h after the first radiation treatment. After the second radiation dose cells were immediately processed for the survival assay.

Cells were irradiated using an X-ray unit operating at 150 kVp with 1 mm aluminum filtration giving a dose rate of 1.2 Gy/min. Hyperthermia was given by immersing the cells sealed in tissue culture flasks into a 40 ± 0.05 °C waterbath for 1 h. At the end of the treatment the flasks were equilibrated in a 37 °C waterbath for 5 min. Hyperthermia was given for 1 h just before or after the first dose of radiation. When both hyperthermia and cisplatin were given after the first dose they were given concomitantly. After all treatments had been completed, the cells were trypsinized and plated for the survival assay.

Cell survival after treatment was determined by a colony-forming assay. Briefly, cells were rinsed with isotonic citrate saline, trypsinized (0.2% w/v trypsin in citrate saline for 5 minutes at 37 °C), counted using an electronic cell counter and plated into 60-mm dishes containing fresh medium. Colonies larger than 50 cells at day 10 and 14 (A2780S and A2780CP, respectively) were scored as survivors. Three replicate dishes were plated for each point. Plotted points represent the mean of three replicate experiments. Error bars are standard error of the mean. Recovery ratios (RRs) were calculated by taking the survival after a specified incubation time and dividing by the survival for zero incubation time. The RRs for combination treatments were compared with those for irradiation alone to determine whether the combination treatment resulted in a reduced RR.

Results

The cisplatin responses of the two cell lines used in this study are shown in Fig. 1. The cells were exposed for 1 h over a wide concentration range and the results show that the $A2780^{cp}$ line was much more resistant to cisplatin than the parental line. The radiation responses of these two cell lines have been reported before (Raaphorst et al. 1995b, 1995c) and indicate that the A2780CP is also more radiation resistant than the parental line.

The data in this study also show that A2780S was more radiosensitive than A2780CP (Fig. 2). When the radiation dose was split into two 4-Gy fractions and incubation was allowed between the fractions both cell lines showed increased recovery as a function of

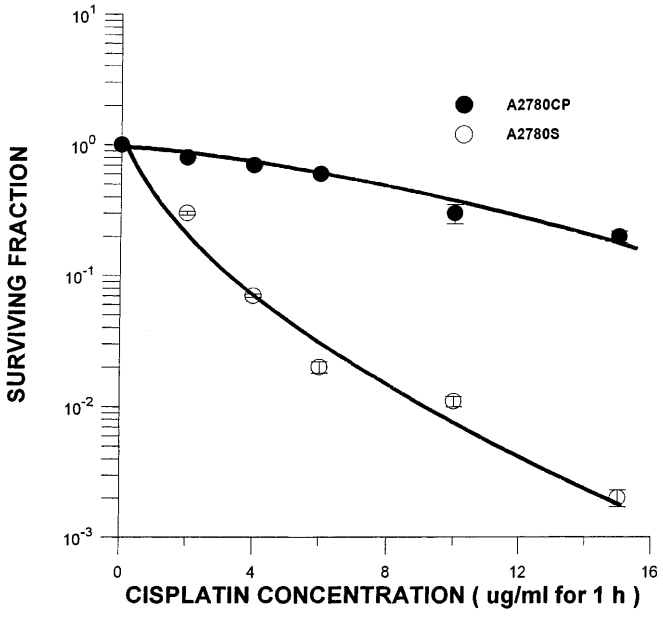

Fig. 1 The responses to cisplatin treatment for 1 h of the two ovarian carcinoma cell lines used in this study are shown

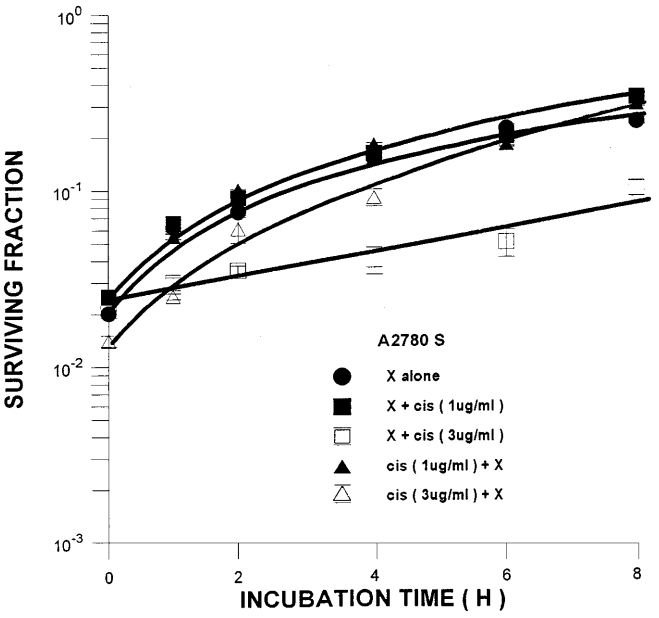

Fig. 3 The effects of cisplatin treatment for 1 h before or after the first of two 4-Gy doses of radiation on SLDR are shown for the sensitive cell line

incubation time. A2780S show more recovery than A2780CP which was probably a result of initially lower survival, thus more damage, which can subsequently be repaired. The RRs at 8 h were 12 and 4 for A2780S and A2780CP, respectively. When 1 h of hyperthermia was given at 40 °C within 5 min after the first dose of irradiation, there was no radiosensitization and recovery was not inhibited.

Figure 3 shows the effect of a 1-h treatment with cisplatin before or after the first dose of the split-dose irradiation protocol for the A2780S cell line. Treatments with 1 g/ml did not affect SLDR. When the concentration was increased to 3 g/ml there was a large inhibitory effect on SLDR when given after irradiation (radiation alone = 12, radiation plus cisplatin = 4.5) while the treatment giving cisplatin before irradiation showed

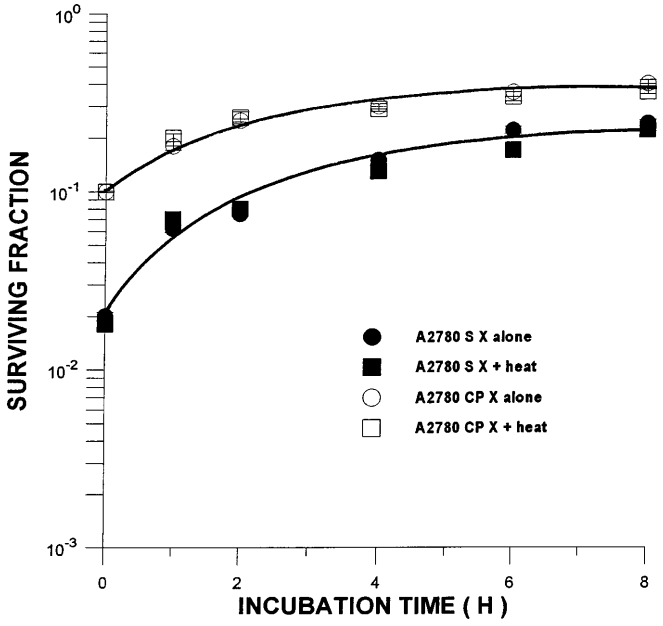

Fig. 2 The recovery from SLD is shown for the two ovarian carcinoma cell. Cells were cultivated at 37 °C between two 4-Gy doses and for hyperthermia a 1-h treatment at 40 °C was given after the first dose

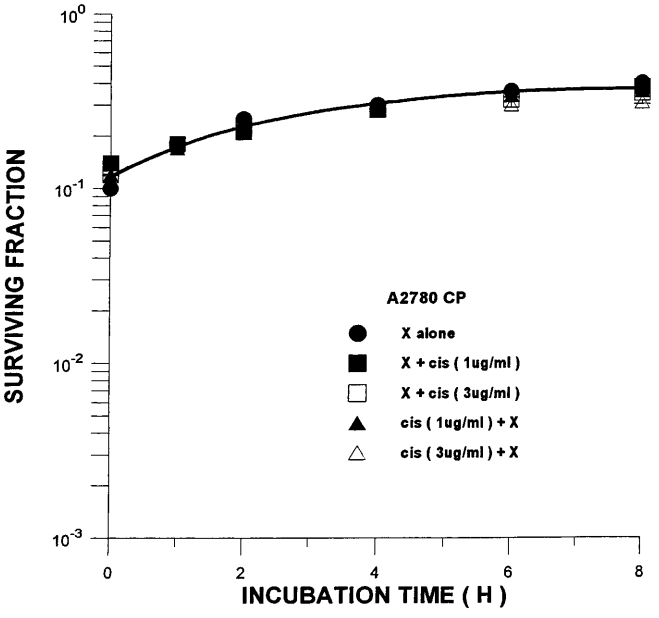

Fig. 4 The effects of cisplatin treatment for 1 h before or after the first of two 4-Gy doses of radiation on SLDR are shown for the resistant cell line

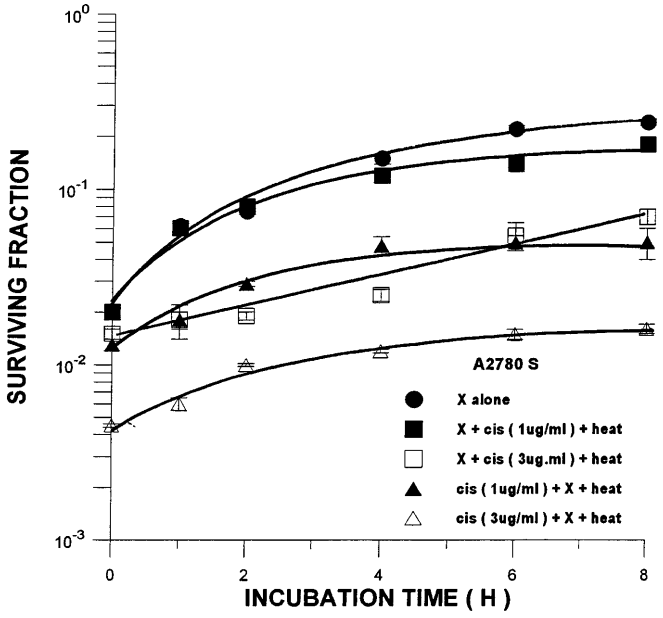

Fig. 5 The effects both cisplatin and hyperthermia (40 °C, 1 h) on SLDR in the sensitive cell line are shown. The sequence of treatments indicated in the figure is with respect to the first dose of the split-dose radiation treatment

only some initial radiosensitization, but SLDR was not reduced. Figure 4 shows the same experiments done in the resistant cell line and the results indicate that neither sequence nor concentration had any effect on SLDR.

The results for the combination of cisplatin treatment and hyperthermia given before or after irradiation are shown in Figs. 5 and 6 for A2780S and A2780CP, respectively. In the parental cell line (Fig. 5) 1 g/ml cisplatin with 40 °C hyperthermia after irradiation had very little effect, while giving the cisplatin before irradiation resulted in some increased toxicity and reduced recovery (RR = 3.9). When 3 g/ml cisplatin treatments were given the sequence cisplatin and hyperthermia after irradiation resulted in reduced recovery (RR = 4.6) while the sequence of cisplatin before irradiation both increased cell killing and inhibited recovery (RR = 3.6). For the resistant cell line A2780CP the effect of cisplatin and hypherthermia combined was very small as shown in Fig. 6. However, cisplatin with hyperthermia after irradiation resulted in reduced recovery (RR 1.5 and 1.2 for the 1 g/ml and 3 g/ml concentrations) while the sequence cisplatin before irradiation had a smaller effect (RR 3 and 3 for the 1 g/ml and 3 g/ml concentrations) compared with the control (RR 4.2).

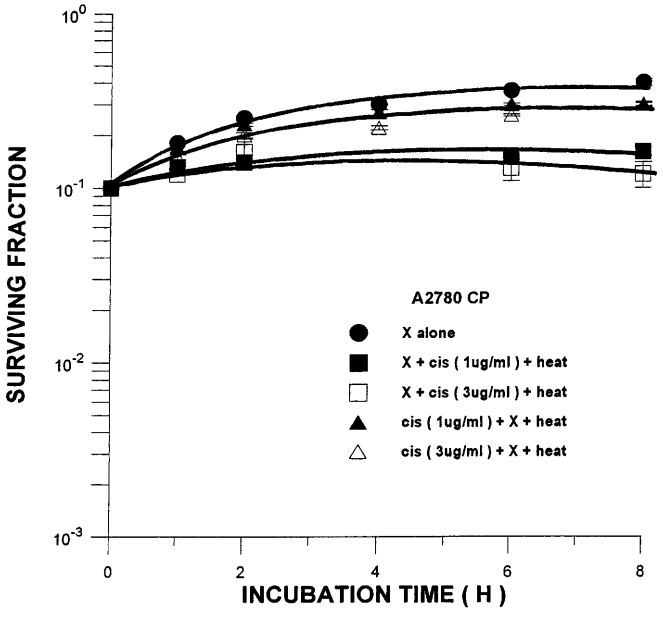

Fig. 6 The effects both cisplatin and hyperthermia (40 °C, 1 h) on SLDR in the resistant cell line are shown. The sequence of treatments indicated in the figure is with respect to the first dose of the split-dose radiation treatment

Discussion

Several studies have shown that radiosensitization by cisplatin treatment is in part a result of inhibition of repair of radiation damage (Dritschilo et al. 1979, Raaphorst et al. 1995; Schwachofer et al. 1991; Wilkins et al. 1993). Our studies with 9L cells have shown that the degree of inhibition of repair is dependent both on the concentration of cisplatin used and on the sequence of treatment (Wilkins et al. 1993). In a recent study with human ovarian carcinoma cells it has been shown that cisplatin treatment can partially inhibit SLDR and that the degree of inhibition is dependent on concentration and treatment sequence (Raaphorst et al. 1995). Others have also shown a sequence dependence (Dewit 1987; Dritschilo et al. 1979; Herman and Teicher 1988; Nias 1985; Schwachofer et al. 1991; Wurschmidt 1992) which may be related to a number of factors such as cell cycle redistribution, thiol depletion and repair inhibition. We have also shown that hyperthermia can inhibit SLDR and that the degree of inhibition is dependent on treatment temperature and sequence (Raaphorst et al. 1979; Raaphorst et al. 1993; Raaphorst et al. 1994). The sequence of heating after irradiation was most effective and we chose this sequence for the present study.

It is well known that high temperature hyperthermia is difficult to achieve uniformly in clinical cancer therapy. In addition, high concentrations of cisplatin for cancer therapy are not only difficult to achieve clinically, but can result in excessive normal tissue damage. Thus a combination treatment of low temperature hyperthermia with cisplatin treatments that result in clinically achievable concentrations may be an effective clinical strategy, especially if combined with irradiation to result in inhibition of repair of radiation damage.

Our results show that, while hyperthermia alone and cisplatin alone (except at the highest concentration in the sensitive cell line) are neither effective in radiosensitization nor in SLDR inhibition, the combination of the two treatments together was effective in both the sensitive and the resistant cell lines. In the sensitive cell line both

increased cell killing and SLDR inhibition were achieved. The treatment sequence of cisplatin before irradiation with hyperthermia after irradiation gave the greatest effect, while SLDR inhibition was evident in both sequences. For the cisplatin-resistant cell line there was no increased toxicity for the combined treatments but there was inhibition of SLDR. This was sequence-dependent and was most effective for the sequence of cisplatin and hyperthermia given after irradiation. The observation that the combined treatments are effective in SLDR inhibition supports the results presented previously indicating that concomitant treatment with mild hyperthermia and cisplatin during low dose rate irradiation is effective in radiosensitization (Raaphorst et al. 1996). It was thought that this may have been through the inhibition of repair of SLDR. The results of the present study directly show that SLDR inhibition occurred for such treatments.

In summary, our results show that the combination of mild hyperthermia and cisplatin treatments may be an effective modality for radiosensitization through inhibition of SLDR and may provide a more effective form of cancer therapy. These treatments were effective in both cisplatin-resistant and -sensitive cell lines and the degree of effectiveness was dependent on treatment sequence which would also have to be considered in clinical application.

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