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The effect of 5-fluorouracil on cisplatin induced DNA interstrand cross-linking in a mouse ascites tumor growing *in vivo*

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In earlier studies we showed that a supra-additive cytotoxic effect was obtained with combined treatment with cisplatin and 5-fluorouracil (5-FU) of Bp8 ascites tumor growing *in vivo* in mice[1]. We therefore studied the DNA interstrand cross-linking after single drug treatment with cisplatin, 5-FU and the drugs combined. The alkaline elution technique was used. 5-FU induced no measurable DNA cross-linking. Cisplatin caused a dose-dependent increase in DNA cross-links 6 h after treatment. When combining cisplatin and 5-FU, no significant change in the number of DNA cross-links was found for this time. The removal of DNA interstrand cross-links was studied after 24 and 36 h. There was no difference in the removal rate between single drug treatment with cisplatin as compared with the combined treatment with the two drugs. Also, the number of DNA single strand breaks (SSB) possibly created when the cisplatin induced cross-links or UV-induced DNA damage were repaired were measured with the alkaline elution technique. No increase in the amount of SSB was seen after combined treatment. Thus, we found no interaction of 5-FU and cisplatin in terms of DNA damage. Other mechanisms for the supra-additive effect found when combining cisplatin and 5-FU have to be considered.

Key words: Cisplatin, cross-linking, DNA, 5-fluorouracil.

Introduction

Treatment of patients with squamous cell carcinoma of the head and neck with cisplatin and 5-fluorouracil (5-FU) induces objective response rates of 70–90%, with 20–30% complete remissions.[2–4] A supra-additive cytotoxic effect when cisplatin and 5-FU is combined remains, however, to be proven in randomized clinical trials. *In vitro* studies of a human colon carcinoma cell line treated with cisplatin and 5-FU in combination or as single drugs showed a supra-additive cytotoxicity by the combination of the drugs.[5] Also in animal tumor systems, supra-additive cytotoxic effects have been found. In L1210 leukemia bearing mice the combination of cisplatin and 5-FU increased the cure rate compared to single drug treatment.[6] We have previously shown a supra-additive cytotoxicity on Bp8 mouse ascites tumor growing *in vivo* after combined treatment with cisplatin and 5-FU. The supra-additivity was demonstrated by growth kinetic studies as well as studies of cell death.[1,7]

Cisplatin is known to produce different types of DNA adducts which it has been suggested are important for the cytotoxicity.[8–11] These are DNA interstrand cross-links, DNA intrastrand cross-links and DNA protein cross-links.[8,11] The vast majority of DNA adducts consists of DNA intrastrand cross-links. Both DNA intrastrand and intrastrand cross-links correlate to cytotoxicity.[8,10,11] There are different mechanisms by which 5-FU can act as a cytotoxic agent. One is by inhibiting DNA synthesis via inactivation of the thymidylate synthase. 5-FU can also be incorporated into RNA with possible negative effects on the protein synthesis, eventually with lethal effects on the cell.[12–16] Incorporation of 5-FdUTP into DNA has also been suggested as a possible mode of cytotoxic action.[16–20]

The supra-additive cytotoxic effect caused by the combination of cisplatin and 5-FU indicates an interaction of the drugs. Other antimetabolites like ara-C and hydroxyurea may increase the cytotoxic effect by modulating the level of cisplatin induced DNA interstrand cross-linking.[21] Therefore, the aim of this study was to elucidate the possible effect of 5-FU on peak concentration and removal of cisplatin induced DNA interstrand cross-linking and the subsequent single strand breaks (SSB).

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Materials and methods

Experimental tumor and animals

Bp8 ascites tumor cells were administered every 10th day by i.p. injection into 3 month old male NMRI mice with a body weight of 20–25 g. Water and standard food were given *ad libitum*. At the start of the experiment 20×10^6 cells in 0.2 ml saline were transplanted after appropriate dilution.

Cytostatic treatment

On day 4 after transplantation, either cisplatin (a gift from Bristol–Myers–Squibb AB, Bromma Sweden) (0.5 mg/ml), 5-FU (25 mg/ml) or the drugs combined were injected i.p. into the mice in a volume of 0.2 ml after proper dilution with normal saline solution. When combining the drugs, cisplatin was given 30 min before 5-FU. The doses of 25, 100 and 200 μg cisplatin/animal, corresponding to about 0.8, 3.35 and 6.7 mg/kg body weight, and 0.9 mg 5-FU/animal, corresponding to about 36 mg/kg body weight, were used.

Measurement of DNA interstrand cross-linking and DNA SSB

The alkaline elution technique developed by Kohn[22,23] was used with minor modifications. Twenty-four hours before cytostatic treatment, tumor cell DNA was radioactively labeled by i.p. injection of [^{3}H]thymidine (5 μCi, specific radioactivity 25 Ci/mmol). Six, 24 and 36 h after treatment the animals were killed by cervical dislocation, and the tumor cells were isolated. The cells were then resuspended in ice cold medium with 4% fetal calf serum and irradiated with 6 Gy for estimation of DNA interstrand cross-links. When SSB were assayed the irradiation was omitted. The cells were then collected on polycarbonate filters (pore size 2 μm, diameter 25 mm; Nucleopore, Pleasanton, CA) and washed twice with 10 ml ice-cold PBS and lysed with 5 ml sarkosyl solution (2% sarkosyl, 0.1 M glycine, 0.025 M Na_2EDTA and adjusted to pH 10.0 with 5 M NaOH). Another 2 ml of sarkosyl solution with 0.5 mg proteinase K/ml was added to each filter. Proteinase K remained in contact with the filters for 1 h. The DNA was then slowly eluted from the filters with a tetraethyl-ammonium hydroxide solution (0.02 M EDTA, 64 ml TEAH/1000 ml, 0.1% sarkosyl, the pH was adjusted to 12.1 with TEAH). The DNA was eluted for 16 h and collected in eight fractions. The radioactivity in the fractions was measured by liquid scintillation. DNA remaining on the filters at the end of the elution was removed by hydrolysis in 0.4 ml 1 M HCl at 60°C for 1 h, followed by treatment with 2.5 ml 0.4 M NaOH at room temperature for 1 h. The activity released from the filters was measured by liquid scintillation counting. DNA remaining in the tunnels, filters, holders and tubes was removed by pumping 2.5 ml 0.4 M NaOH through the system. The activity obtained was added to the activity released from the filters. The number of DNA cross-links was calculated according to the formula developed by Kohn.[23]

UV irradiation

Tumor cells (0.6×10^6) in 2 ml PBS + 5% fetal calf serum, prelabeled with [^{3}H]thymidine (5 μC, specific activity 25 Ci/mmol) for 24 h, were irradiated in glass Petri dishes (diameter 5 cm) with a dose of UV of 19.2 J/m^2. The dose rate was 0.32 J/m^2/s. After irradiation, the cells were washed once, resuspended in Eagle's medium containing 1 mM L-glutamine, 10% fetal calf serum with or without 5-FU and incubated at 37°C. In some experiments, the cells were pre-treated with 5-FU for 24 h, and then irradiated with UV as described above and further incubated for 2 h.

Results

DNA interstrand cross-links

Bp8 ascites tumor was treated *in vivo* in mice with 0.8, 3.3 and 6.7 mg cisplatin and 36 mg 5-FU/kg body weight either as single drugs or in combination. DNA interstrand cross-linking was measured with the alkaline elution technique at 6, 24 and 36 h after treatment. Since it has been shown that the peak concentration of DNA interstrand cross-links appears 6–12 h after treatment, the first point of investigation was after 6 h.[24–26]

Cisplatin treatment resulted in a dose-related increase in the concentration of DNA interstrand cross-links at 6 h. After treatment with 0.8 mg/kg body weight of cisplatin, the number of cross-links corresponded to 0.10 Gy equivalents. The doses of 3.3 and 6.7 mg cisplatin/kg body weight increased DNA cross-linking to 0.85 and 1.60 Gy equivalents, respectively (Figure 1). After single drug treatment with 36 mg/kg body weight 5-FU, no detectable

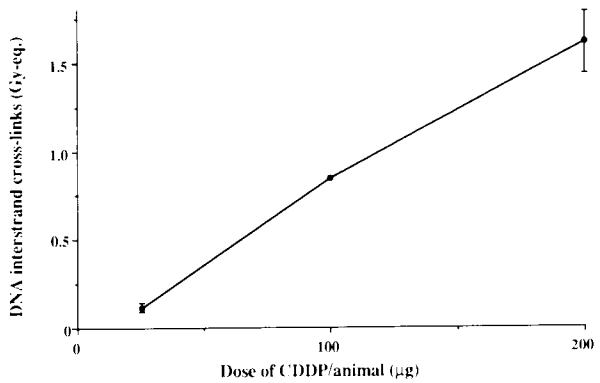

Figure 1. DNA interstrand cross-links in Bp8 mouse sarcoma cells in relation to drug concentration 6 h after treatment with cisplatin. The drug was i.p. injected into the animals. Mean values ± SE of four mice.

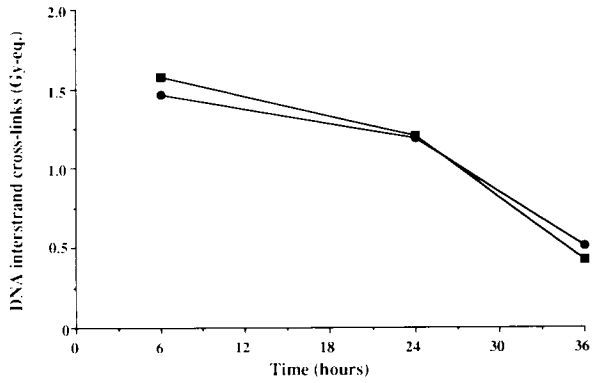

Figure 2. DNA interstrand cross-links in Bp8 mouse sarcoma cells 6, 24 and 36 h after treatment with 200 μg cisplatin/animal (●), and in combination with 0.9 mg 5-FU (■). Cisplatin was i.p. injected into the mice 30 min prior to the 5-FU. Mean values of two mice.

DNA interstrand cross-linking was found (data not shown).

For combined treatment with the two drugs, 6.7 mg cisplatin/kg body weight was chosen together with 36 mg 5-FU/kg body weight. This 5-FU dose was chosen since it caused a supra-additive cytotoxic effect in combination with cisplatin on the same tumor system.[7] No significant difference in the levels of DNA interstrand cross-linking were observed between cells treated with cisplatin and cells treated with cisplatin and 5-FU. The number of DNA interstrand cross-links 6 h after combined treatment corresponded to 1.50 Gy equivalents. There was a gradual decline in the DNA cross-linking over the observation period. At 24 h the level corresponded to 1.20 Gy equivalents and at 36 h to 0.50 Gy equivalents. Thus, no sigificant differences in the peak concentration or in the rate of removal of the DNA interstrand cross-links were seen when single drug treatment and treatment with the drug combination were compared (Figure 2).

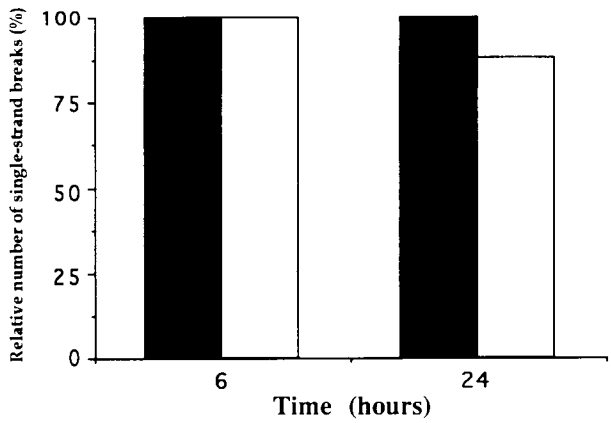

Figure 3. DNA SSB in Bp8 mouse sarcoma cells as measured by the alkaline elution technique of untreated cells (■) and 6 and 24 h after treatment with 200 μg cisplatin/animal combined with 0.9 mg 5-FU/animal (□). Cisplatin was i.p. injected into the mice 30 min prior to the 5-FU. Results of one typical experiment. The values are depicted relative to the control values, which were given the value 100%.

DNA SSB

Although we obtained no difference in the removal of cross-links after combined treatment as compared to single drug treatment with cisplatin, it is still possible that 5-FU may interfere with the repair of cisplatin induced DNA damage.

Cells were therefore treated *in vitro* with 36 mg kg 5-FU and 0.8 mg kg cisplatin, and 6, 15 and 24 h later SSB were determined by the alkaline elution technique. No major differences in the frequency of SSB were found between the combined and the single drug treatment (Figure 3).

However, cross-links may mask the appearance of SSB following the repair of the cross-links and thus make them undetectable with the techniques described above. In order to elucidate this possibility, SSB was induced by UV irradiation *in vitro* and the frequency of SSB, as measured by the alkaline elution, was followed in the presence of 20 μM 5-FU up to 6 h. No difference in the frequency of SSB was found between cells treated with or without 5-FU (Figure 4A). However, when the cells were pretreated with 5-FU for 24 h and then UV irradiated, an increased level of about 25% SSB was observed (Figure 4B).

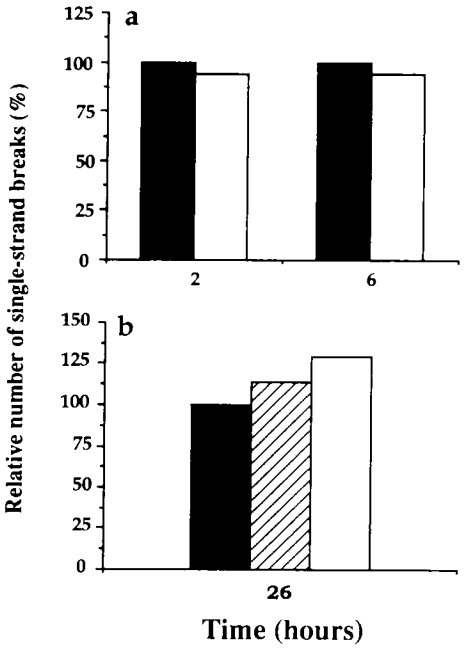

Figure 4. DNA SSB of Bp8 mouse sarcoma cells after UV irradiation with a dose of 19.2 J/m^2 in combination with 5-FU expressed as a percentage of UV irradiated cells (■). SSB were measured by the alkaline elution technique. (a) Cells were UV irradiated *in vitro* and then treated with 20 μM 5-FU (□) for 2 and 6 h. (b) Cells were pre-treated with 10 μM (▨) and 20 μM 5-FU (□) for 24 h, followed by UV irradiation and then, after a further 2 h, SSB of the cells were determined. Results of one typical experiment. The values are depicted as relative to the control values, which were put to 100%.

Discussion

After combined treatment with cisplatin and 5-FU, a supra-additive cytotoxic effect has been found in different tumor systems.[1,5,6] Cisplatin is known to produce DNA interstrand, DNA intrastrand and DNA protein cross-links.[8,10,11] 5-FU acts by inhibiting DNA synthesis via inhibition of the thymidylate synthase, direct incorporation into RNA and by direct incorporation of FdUTP into DNA.[27] One possible explanation for the supra-additive toxic effect seen after combined treatment with 5-FU and cisplatin may be inhibition of the repair of cisplatin induced monoadducts and/or bifunctional adducts such as DNA interstrand cross-links. This would increase the formation and/or decrease the removal of bifunctional adducts and thus decrease the ability for the cell to survive. A combination of ara-C and hydroxyurea was used together with cisplatin.[21] In those experiments it was possible to demonstrate a synergistic effect on colony formation in an HT-29 human colon carcinoma cell line growing *in vitro*. There was a correlation between the cytotoxic effect and the number of DNA interstrand cross-links found by alkaline elution.

We used the alkaline elution technique to detect the DNA interstrand cross-links. The majority of cisplatin induced DNA adducts consists of DNA intrastrand cross-links, but the DNA interstrand cross-links correlate well to cytotoxicity.[9,28] DNA interstrand cross-links accumulate 6–12 h after a cisplatin pulse treatment and at later time points the cross-links level off.[24–26]

We found a dose-dependent increase in the concentration of DNA interstrand cross-links at 6 h after cisplatin treatment. On the other hand, 5-FU did not produce any detectable DNA interstrand cross-links or DNA SSB (data not shown). At a dose of 6.7 mg cisplatin/kg of body weight a significant concentration of DNA interstrand cross-links was found at 6 h. When combining cisplatin with 5-FU we found no significant increase in the concentration of DNA interstrand cross-links at this time point. To further study the effect of 5-FU on the repair process, we analyzed the concentration DNA interstrand cross-links at 24 and 36 h after cisplatin treatment as a single drug or in combination with 5-FU. No significant change in the removal rate of the cisplatin induced DNA interstrand cross-links was found after the combined treatment.

Synergistic effects of cisplatin in combination with ara-C have been reported.[29,30] It has not been possible to clearly correlate this synergy with an increase in DNA interstrand cross-links.[31] The reason for the difference in results between those experiments and ours, on the one hand, and experiments with cisplatin and a combination of ara-C and hydroxyurea, on the other hand, is unknown.[21] The combination of ara-C and hydroxyurea does in itself have synergistic effects and the mechanism responsible for the cytotoxic effect is most probably different from that of 5-FU, making a comparison between the treatments difficult.

The unchanged levels of DNA cross-links found in our experiments do not exclude an effect of 5-FU on the excision repair of DNA damage with subsequent fragmentation of the DNA molecule.[32] The importance of the dose of 5-FU used cannot be disregarded. An increase in the dose of 5-FU can change the mechanism of action of 5-FU in combination with cisplatin. We conclude, however, that the supra-additive cytotoxic effect earlier shown by us when combining 36 mg 5-FU/kg of body weight and 0.8 mg cisplatin/kg of body weight cannot be the result of an interference of 5-FU with the concentration of DNA interstrand cross-links.

Other mechanisms responsible for the supra-additive cytotoxicity must also be considered. Unbalanced growth, resulting in changes in the protein/DNA ratio and/or changes in the intracellular concentration of deoxyribonucleotide triphosphates[33,34] may cause DNA strand breaks resulting in cell death if not repaired.[35] During the repair of cisplatin induced DNA cross-links SSB are generated. We did, however, not observe any increase in the number of SSB following combined treatment when cisplatin was administered before 5-FU. On the other hand, in another experiment we saw an increase in the number of SSB generated when the cisplatin treatment was given after the 5-FU (data not shown).

In earlier studies in which we demonstrated a supra-additive cytotoxic effect, a parallel inhibition of the DNA synthesis rate was found after treatment of the Bp8 cells with 5-FU or cisplatin as single drugs. Combination of the drugs resulted in a partial restoration of the DNA synthesis rate.[7] This relative increase in the DNA synthesis rate may have misincorporation of deoxyribonucleotides into DNA as a possible consequence.

We thus conclude that the supra-additive cytotoxic effect observed after combined treatment of Bp8 sarcoma cells with 5-FU and cisplatin in a limited sequencing is not caused by modulation of the cisplatin induced DNA interstrand cross-links by 5-FU or any increase in the number of SSB. Other possible mechanisms are at present under evaluation.

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