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Persistence of cisplatin-induced DNA interstrand crosslinking in peripheral blood mononuclear cells from elderly and young individuals

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Abstract DNA interstrand crosslinks (ISCL) produced by the incubation of human peripheral blood mononuclear cells (PBMs) with cisplatin (CDDP) were measured in two populations of volunteers, one aged less than 25 years and the other, greater than 72 years. The technique of fluorometric alkaline elution was used to measure DNA interstrand crosslinking with time after a 1-h exposure to drug. The samples from the young group showed a more consistent pattern of crosslink formation and removal, with extensive, or in some cases complete, repair at 48 h. Those from the elderly group showed considerable inter-individual variation and significantly higher mean levels of crosslinking at 24 and 48 h. No samples showed complete repair at 48 h in this population. These results indicate an impaired DNA repair capacity in the cells from the elderly group. This may be a factor in the poor tolerance of chemotherapy in the ageing population

Key words Cisplatin · DNA crosslinking · DNA repair · Ageing

Introduction

It is a commonplace of cancer chemotherapy that toxicity increases with age yet most tumours occur in the elderly, with about 50% of all solid tumors presenting in patients over 70 years [1]. Many clinical trials exclude patients over a certain age, and most studies do not report response rates and side effects related to age. There has been little systematic investigation of the reasons for increased toxicity, despite this problem being the basis for decisions about treatment with chemotherapy in the elderly.

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Cisplatin is used widely for the treatment of solid tumours. It is the first-line single-agent treatment for carcinoma of the ovary [2] and is widely used in treatment of testicular [3], non-small-cell lung and bladder cancers. The main early toxicity is vomiting, and later toxicity can be severe, causing neuropathies and renal failure [4, 5]. The major cytotoxic target of cisplatin appears to be DNA. The type of DNA lesion responsible for the cytotoxicity and antitumour activity, however, is not clearly established. The cytotoxicity of cisplatin in cultured cells has been found to be directly related to total platinum binding [6, 7] interstrand crosslinking [8, 9], and intrastrand crosslinking at d(GpG) and d(ApG) sites [10]. The relative percentages of these lesions have been studied in isolated DNA [11], DNA isolated from drug-treated murine cells [12] and in leukocyte DNA from cancer patients receiving cisplatin-based chemotherapy [13, 14]. In each case the relative percentages were similar, with intrastrand adducts accounting for approximately 90% of lesions, interstrand crosslinks less than 1%, and the remainder the result of a variety of lesions, including DNA–protein crosslinks and monoadducts. Increased DNA repair capacity plays a major role in resistance to cisplatin in several mammalian cell lines, including human ovarian and bladder cells [12, 15, 16].

The present study investigates the onset and disappearance of crosslinks in the peripheral blood mononuclear cells from either young (< 25 years) or elderly (> 72 years) volunteers exposed in vitro to doses and durations of cisplatin achievable clinically.

Materials and methods

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMs) were isolated from two populations of individuals. The elderly group (mean age 75 years, age range 72–81 years) was made up of patients admitted to the orthopaedic wards for surgical operations, who were otherwise fit

and not taking any significant medication. Blood was taken pre-operatively and with informed consent. The younger group (mean age 24, age range 21–25 years) were laboratory staff, nurses or medical students, who were fit and not taking any medication. A sample of 30 ml was taken from each person, and PBMs were isolated using the ficoll-hypaque method, which included washing four times with MEM containing 10% fetal calf serum (FCS) before incubation in the same medium. Each experimental point was performed in triplicate.

Drug treatment in vitro

Cisplatin (Sigma Chemical Company) was dissolved in serum-free MEM by heating to 37°C for 30 min immediately prior to use. Where appropriate the PBMs were exposed immediately to drug for 1 h at 37°C. Cells were then washed twice in drug-free medium, resuspended in fresh medium, and reincubated for the requisite post-incubation time. Cell viability was assessed using trypan blue exclusion and cells counted on a haemocytometer at each time point. Viability always exceeded 90%.

Measurement of DNA interstrand crosslinks

A modification of the alkaline elution technique of Kohn [17] was used, in which the DNA is quantitated fluorometrically [18]. After appropriate post-incubation times, PBMs were chilled on ice and irradiated to introduce random DNA single-strand breaks (6Gy, dose rate 4.5 Gy/min). They were then deposited onto polycarbonate filters (Nucleopore 25 mm diameter, 0.2 μM pore size) pre-chilled with cold PBS at $1.5–2.5 \times 10^6$ cells per funnel. The funnel outlet was plugged and cells were lysed with 2 ml sarcosyl lysis solution (0.2% sarcosyl, 0.4 M EDTA-Na_2, 2 M sodium chloride, pH 10) containing 0.5 mg/ml proteinase K (Sigma) for 20 min. The funnels were then unplugged, allowing the lysis solution to drip through. The DNA was washed with 10 ml of 0.2 M EDTA-Na_2 pH 10. After washing, the funnels were then connected to the pumps and the DNA was eluted at a constant rate of 2 ml/h with elution buffer (tetraethylammonium hydroxide, 0.02 M EDTA-Na_2, pH 12.2). Five 6-ml fractions were collected over a 15-h period.

After this, 3 ml of each of the fractions was adjusted to pH 6.8–7.1 by the addition of 1 M potassium phosphate. The filters were removed from the funnels and incubated at 55°C for 1 h in elution buffer and then vortexed rapidly for 2 min. To each 3 ml sample, 0.8 ml 2.25 μM Hoechst 33258 was added, and after mixing the samples were allowed to stand for 30 min at room temperature. The fluorescence was then read on a fluorimeter (Perkin Elmer Model LS-2B) at an excitation of 350 and emission 475 nm. A blank elution lane was included to adjust for background fluorescence, and the values adjusted for the volume of each sample. A standard curve of fluorescence was constructed using calf thymus DNA in the same buffer. The fraction of DNA remaining on the filter was calculated at each time point and elution curves constructed from control and drug-treated cells. The crosslink index was calculated at 12 h of elution according to the formula:

$$\text{Crosslink index (in rad equivalents)} = [\sqrt{(1 - R_0)/(1 - R)} - 1] \times 600$$

where R_o and R are the relative retentions for the control and drug-treated irradiated cells, respectively. In each experiment control lanes containing DNA from unirradiated and irradiated human K562 cells were run to ensure consistency.

Results

Cisplatin-induced DNA ISCL were measured in PBMs from young and elderly volunteers following a 1-h exposure to the drug. Initial experiments were performed at a dose of 50 μM in 8 young and 7 elderly patient samples. Crosslinking was measured at 6, 24 and 48 h after incubation. In the samples from young patients the range of the cross-link indices observed at 6 h was 0–71. At 24 h fewer crosslinks were detected than at 6 h in all samples except one, and in half of the samples no crosslinks were detectable. By 48 h no crosslinks were detected in any sample. In the PBMs from the 7 volunteers over 72 years the crosslink indices ranged from zero to 55 at the 6-h time point. At 24 h 4 had an increased number of crosslinks and only 2 had undetectable levels. By 48 h post incubation only 3 had no detectable crosslinking.

Despite considerable inter-individual variation the data from this small sample number suggested distinct differences between the two populations and suggested that there might be differences in the cellular processing of DNA interstrand crosslinks between the two groups. This prompted a larger study with the higher concentration of 70 μM cisplatin, in order to maximise any differences between the two age groups. In this study, 12 volunteers were studied in each age group and crosslink levels were again measured at 6, 24, and 48 h following a 1-h treatment. The data from the young volunteers are shown in Fig. 1. At 6 h the range of crosslink indices was 14–73. In 6 samples more crosslinking was observed at 6 h than at 24 h, whereas in the other 6 increased levels were observed at the 24-h time point. In all samples, however, the number of crosslinks was smaller at 48 h than at 24 h. In 2 samples there was complete repair by 48 h, and in 11 of the 12 samples the crosslink index was less than 20 following this dose of cisplatin.

The results in the 12 samples from the elderly volunteers (Fig. 2) show considerable inter-individual variation, which is more marked than in the young group.

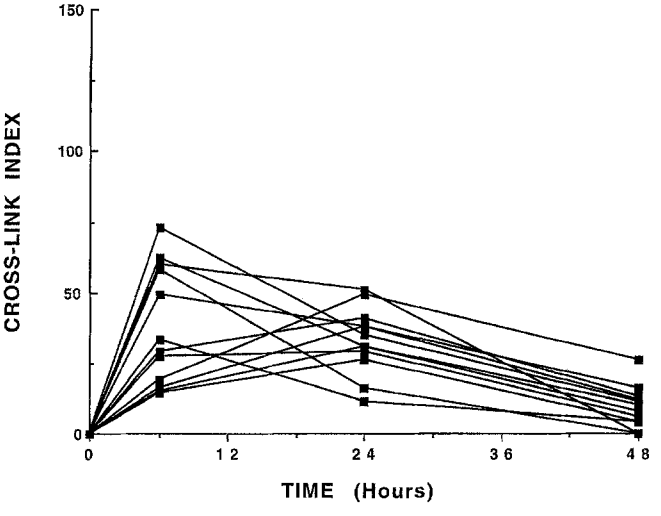

Fig. 1 DNA interstrand crosslink profiles in PBMs obtained from young volunteers and incubated with 70 μM cisplatin for 1 h

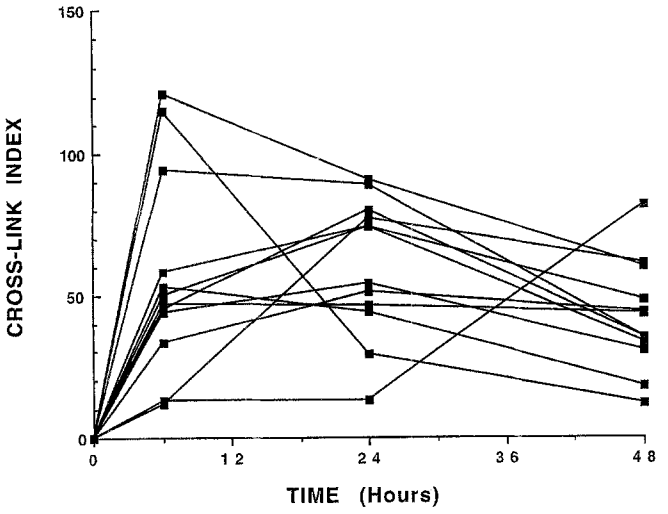

Fig. 2 DNA interstrand crosslink profiles seen in PBMs obtained from volunteers over 72 years of age and incubated with 70 μM cisplatin for 1 h

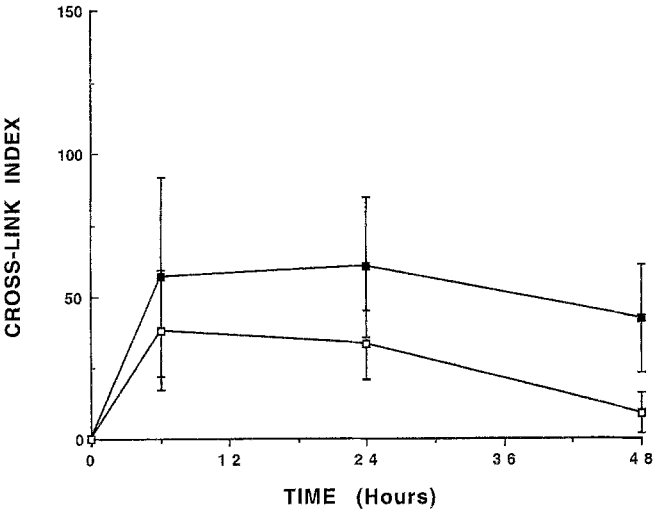

Fig. 3 Combined DNA interstrand crosslink profiles from both the elderly (*closed symbol*) and the young (*open symbol*) groups

At 6 h the crosslink index varied widely (12–121), with 3 samples lying outside the range observed in the young group. In 5 the crosslinking was higher at 6 h than 24 h and in a further 6 samples the level was higher at 24 h. All 11 had fewer crosslinks at 48 h. None, however, had completely repaired the crosslinks at 48 h, and only 2 had crosslink indices below 20. There was 1 unusual profile that had low levels at 6 and 24 h and then increased crosslinking at 48 h.

The results from the two age groups are summarised in Fig. 3. There was no statistically significant difference between the two age groups at 6 h, but significant differences were observed at 24 h ($P < 0.01$) and 48 h ($P < 0.001$).

Discussion

In this study interstrand crosslinks were measured and compared in the two groups of healthy people at the extremes of adult life. Although the levels of interstrand crosslinking adducts were not significantly different between the two groups at 6 h they persisted for longer periods in the elderly group than in the young. Since the adduct levels were similar at 6 h, cellular detoxification mechanisms such as glutathione are unlikely to be a major factor. In both groups the peak of crosslinking was observed between 6 and 24 h. This is consistent with other cellular studies, which show a peak at 6–12 h [8, 9]. The peak level of crosslinking in the PBMs from young individuals appears somewhat lower than that in cells from elderly subjects. This suggests the possibility that the young cells have a more efficient mono-adduct repair. The level of crosslinking observed with the in vitro treatment is within the range achieved clinically; in several experiments in which PBMs were isolated directly from patients following a cisplatin infusion (80 mg/m^2) the levels of interstrand crosslinks observed fell within the range observed in the present study (data not shown).

It is not certain whether DNA repair declines as animals age, however, no DNA repair system is 100% efficient, and it is therefore possible that cells may accumulate DNA damage with ageing [19]. DNA repair may become less efficient with age, and this loss of DNA repair may itself contribute to the aging process [19]. Studies of DNA repair after chemical or UV damage in cultured cells have shown conflicting results and given no clear indication that DNA repair does decline with cell age. Results obtained in animal studies are also varied, but some studies have shown some deficit in repair. For instance, post-mitotic liver parenchymal cells from aged rats exposed to carcinogens have loss of repair activity [20], and more competent repair of ethylnitrosurea has been seen in young fibroblasts than in old ones [21]. Reduced survival of lymphocytes from patients over 60 years has been observed after exposure to X-rays [22], and Prieur [23] has described increased numbers of chromosomal and chromatid lesions in lymphocytes from elderly people. Most chemical studies have involved low doses of a carcinogen and have examined either the induction of cancers or the efficacy of DNA repair in ageing cells or cell lines. There is little information on the effect of ageing on the severe degree of DNA damage induced by drugs such as cisplatin. It is possible that repair mechanisms of cells in old age are deficient on exposure to extensive cytotoxic drug damage.

In studies of cisplatin-induced DNA damage cytotoxicity has been correlated with interstrand crosslinking, intrastrand crosslinking and levels of platination [6–10]. Controversy remains over which type of DNA adduct is responsible for the cytotoxicity of cisplatin, although the more numerous intrastrand

crosslink seems more likely to be the major lesion. Interstrand crosslinks account for approximately 1% of all adducts. Several studies have correlated levels of ISCL with cytotoxicity [6, 7]; however, it is possible that this merely reflects a similar relationship between the formation of interstrand and intrastrand crosslinks [24]. A correlation has been shown between formation of intrastrand crosslinks and tumour response in patients receiving cisplatin for ovarian and testicular tumours [25, 26]. More recently it has been suggested that acquired cellular resistance to cisplatin may be associated with increased gene-specific DNA repair efficacy of ISCL but not intrastrand crosslinks [27].

The present study demonstrates that the difference observed in adduct numbers is an event after the formation of the crosslink, and it can therefore be assumed that there is a deficit in the processing of the damage. Further studies will be needed to determine whether the deficit lies in one step or in a general reduction in all the enzymatic steps in repair. Additional studies are necessary to determine whether there is persistence of intrastrand crosslinks in the elderly.

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