Reduced DNA Damage Induced in Human Melanoma Cells by Dacarbazine in the Presence of Deoxyribonucleosides

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ABSTRACT

Dacarbazine induces DNA lesions in replicating DNA. We show here that deoxyribonucleosides, with the exception of thymidine, in doses of 250 μM or higher, prevent dacarbazine-induced DNA lesions. The DNA lesions that appear in the presence of thymidine can be prevented by aphidicolin, an inhibitor of DNA synthesis. Cytotoxicity analyses confirm that deoxyribonucleosides, with the exception of thymidine, in doses of partially, while other deoxyribonucleosides completely abolish the dacarbazine effect.

INTRODUCTION

The antineoplastic agent dacarbazine (DTIC-Dome; 5-3,3-dimethyl-1-triazeno)imidazole-4-carboxamide) is a purine analogue which is frequently used in the treatment of disseminated human malignant melanoma (1). The main mechanism of action of this drug remains unclear; however, there are two possible pathways. The relative importance of each pathway is at present not known.

One pathway is the induction of DNA lesions either due to the incorporation of the drug into DNA or attack of the drug on the single-stranded DNA transiently present during DNA synthesis (2). The appearance of DNA lesions can be prevented by stopping DNA synthesis (2) and augmented by inhibiting either calmodulin (a Ca²⁺-binding protein) (3) or poly(ADP)-ribose synthetase (4).

The other pathway involves the metabolism of the drug by microsomal enzymes (oxidative N-demethylation) giving rise to metabolites showing cytotoxic activity (5–9), presumably due to an ability to alkylate DNA. 7-Methylguanine is the predominant product, as has been shown both in cultured cells and in animals. However 7-methylguanine is unlikely to be crucial for cytotoxicity. Instead the formation of O⁶-methylguanine probably plays a more important role (10).

In this paper we analyze whether exogenously added purine and/or pyrimidine deoxyribonucleosides may protect the cells from the dacarbazine-induced DNA damage. It is known that mammalian cells contain kinases active in the phosphorylation of deoxyribonucleosides and that the phosphorylation of deoxycytidine, deoxyguanosine, and deoxyadenosine is catalyzed by the same enzyme (11).

We show here that deoxyadenosine, deoxyguanosine, and deoxycytidine but not thymidine prevent dacarbazine from inducing DNA damage.

MATERIALS AND METHODS

Cells, Culture Methods and Labeling with [³H]Thymidine. A human melanoma cell line (CRL 1424) was grown as monolayers as earlier described (2). For experiments involving prelabeled DNA the cells were seeded in small culture dishes (35 x 10 mm) containing 3-ml medium. [³H]Thymidine (30 μCi; 20 Ci/mmol; Amersham Inc.) was added to the culture medium. After 24 h the medium was changed to fresh medium without thymidine and after another 24 h the cells were used for drug treatment experiments.

Survival of drug-treated cells was determined by the out-growth method described by (12).

The deoxyribonucleosides were obtained from Sigma Biochemicals. Aphidicolin was obtained from Boehringer Inc.

Dacarbazine was obtained from a local pharmacy. Fresh solutions were always made up immediately before experiments. All our experiments were performed in the dark since degradation may occur during light conditions (8).

Cell Lysis. For cell lysis the incubation medium was drained off and 2.25 ml of 0.03 m NaOH was added. After 30 min at 0°C in the dark the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl

Gel Electrophoresis. The labeled DNA was separated in 0.75% agarose gels using a LKB Multiplier electrophoretic system. After the separation was terminated the gels were sliced in 1-mm-thick slices. The slices were placed in scintillation fluid containing 3% Soluene 100 (Packard) and the radioactivity was measured in a Packard scintillation counter.

RESULTS

Approach. To visualize the appearance of DNA lesions we lysed the cells in dilute alkali to partly denature the DNA (13, 14). This treatment removes macromolecules from DNA and disrupts the base pair structure of the DNA. However the DNA strands cannot separate until enough time has passed to allow unwinding. Unwinding is initiated at gaps/alkali-labile regions present in the DNA chains. Such gaps are known to exist during synthesis of new DNA. Further treatment with various drugs such as dacarbazine, 5-fluoropyrimidines, or arabinosylcytosine induces gaps and/or alkali-labile regions in the DNA, resulting in increased number of points at which unwinding can be initiated (2, 3, 15). The length of DNA that can be unwound at each point has been estimated at 20 kilobases (16).

When the alkaline solution is neutralized, the high molecular weight DNA renatures and forms double-stranded DNA. Small DNA fragments induced by the drug-treatment which are released during the unwinding remain in solution as single-stranded DNA molecules. The fragments can then be separated from the high molecular weight DNA by agarose gel electrophoresis.

Dacarbazine Induces DNA Lesions. Cells with prelabeled DNA were treated with dacarbazine (10 μg/ml) for 60 min and the DNA examined either immediately or after incubation in fresh medium for 24 h. The cells were lysed in dilute alkali and the DNA then separated in an agarose gel. The maximum concentration of dacarbazine in plasma of human melanoma patients receiving conventional treatment with dacarbazine is 10 μg/ml (17).

Fig. 1 shows that dacarbazine induces lesions in prelabeled DNA which shows up as the appearance of DNA fragments at
Reduced DNA damage induced by dacarbazine in deoxyribonucleosides

Fig. 1. Treatment with dacarbazine. Human melanoma cells with prelabeled DNA were treated with dacarbazine (10 μg/ml) for 60 min. The cells were then either immediately lysed in dilute alkali (○) or analyzed after incubation in fresh medium for another 24 h (□). Cells not incubated with dacarbazine (⊗). The DNA was separated in 0.75% agarose gels. 25, 10, and 2 denote the sizes (in kilobases) and location of single-stranded DNA markers.

Slices 22–32. The fragments are not detected in untreated cells. Furthermore the level of DNA fragments is higher in cells examined 24 h after the drug treatment. The results are the same as described earlier in ref. 2.

Incubation with Purine Deoxyribonucleosides and Dacarbazine. Dacarbazine is a purine analogue and therefore we tested whether deoxyadenosine or deoxyguanosine may protect the cells from the DNA-damaging effect of dacarbazine.

Cells were incubated with deoxyadenosine or deoxyguanosine (500 μM) for 30 min, dacarbazine then added together with the purine for 60 min, after which the cells were incubated for another 60 min with deoxyadenosine or deoxyguanosine only. The cells were then either immediately lysed or lysed after incubation for 24 h or 48 h in fresh medium.

The gel electrophoretic separations showed, in all experiments involving purine deoxyribonucleosides, only high molecular weight DNA. We did not detect any fragmentation of the DNA. Fig. 2, A–B, shows the data for cells incubated in fresh medium for 48 h. Hence the addition of exogenous purines prevents dacarbazine from inducing DNA lesions.

We have also performed experiments with different concentrations of dacarbazine. Fig. 2C shows that the protective effect of deoxyadenosine increases gradually with increasing concentration. From 250 μM upwards there is complete protection. The results are the same irrespective of which deoxyribonucleoside that was used.

Incubation with Pyrimidine Deoxyribonucleosides and Dacarbazine. To examine the influence of pyrimidines cells were incubated for 30 min with thymidine or deoxycytidine (500 μM) for 60 min, deoxyadenosine and dacarbazine (10 μg/ml) for 60 min, deoxyadenosine for 60 min and then lysed in dilute alkali after incubation in fresh medium for 48 h (○). Cells treated with dacarbazine for 60 min and then incubated in fresh medium for 48 h (□). The DNA was separated in 0.75% agarose gels. 25, 10, and 2 denote the sizes (in kilobases) and location of single-stranded DNA markers. B, the same protocol as in A but deoxyguanosine was substituted for deoxyadenosine. C, the same protocol as in A but the concentration of deoxyadenosine was either 50 μM (○), 100 μM (□), or 250 μM (⊗).
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Fig. 3. Incubation with pyrimidine deoxyribonucleosides and dacarbazine. A, cells with prelabeled DNA were incubated for 60 min with deoxycytidine (500 μM), for 60 min with deoxycytidine and dacarbazine (10 μg/ml), for 60 min with deoxycytidine and for another 48 h in fresh medium (X). Cells treated with dacarbazine for 60 min and then incubated in fresh medium for 48 h (X). The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. 25, 10, and 2 denote the sizes (in kilobases) and location of single-stranded DNA markers. B, the same protocol as in A, but thymidine was substituted for deoxycytidine. However, the cells were lysed after incubation in fresh medium for either 24 h (O) or 48 h (X). C, the same protocol as in B, but aphidicolin (10 μg/ml) was added with the thymidine.

Fig. 4. Outgrowth experiments. A, cells were treated with deoxyadenosine (500 μM) for 60 min, deoxyadenosine and dacarbazine (10 μg/ml) for 60 min, deoxyadenosine for 60 min and then incubated in fresh medium for 48 h (X). The same protocol but deoxyguanosine substituted for deoxyadenosine (D). Cells treated with dacarbazine only for 60 min and then incubated in fresh medium for 48 h (X). Control cells (---). Bars, SEM of three experiments. B, the same protocol as in A, but the purines were substituted for deoxycytidine (D) or thymidine (X).
after incubation for 24 or 48 h.

The gel electrophoretic separations showed that deoxycytidine prevented dacarbazine from inducing DNA lesions. Fig. 3A shows the data for cells incubated in fresh medium for 48 h.

However, thymidine did only partly rescue the cells. DNA fragmentation was detected and it increased with the duration of the postincubation in fresh medium. Fig. 3B shows the data for cells incubated in fresh medium for either 24 h or 48 h. It was not possible to prevent the appearance of DNA lesions by using higher doses of thymidine.

Hence thymidine differs from the other purine deoxyribonucleosides in not being able to completely revert the dacarbazine effect.

Next we wished to examine whether the DNA lesions induced by dacarbazine are of the same type as can be prevented to appear by a pretreatment with aphidicolin (2), an inhibitor of DNA polymerases α and δ (18, 19). Aphidicolin (10 µg/ml) was therefore added with the thymidine but the protocol otherwise not changed. Fig. 3C shows that aphidicolin prevents the damage induced in DNA by dacarbazine in the presence of thymidine.

Cytotoxicity Analysis. Cytotoxicity analyses were performed using an outgrowth method (12). Fig. 4 shows that the addition of deoxyadenosine, deoxyguanosine, or deoxycytidine, but not thymidine, prevents the growth-inhibitory effect of dacarbazine. The combination thymidine-dacarbazine shows a partial growth-inhibitory effect.

DISCUSSION

In this paper we have analyzed the influence of deoxyribonucleosides on the ability of dacarbazine to induce DNA lesions. We find that deoxyadenosine, deoxyguanosine, and deoxycytidine at 250 µM, but not thymidine, prevent dacarbazine from inducing DNA lesions. Even by using higher concentrations of thymidine it is not possible to completely prevent the DNA lesions. In agreement cytotoxicity analyses show that the addition of deoxyribonucleosides with the exception of thymidine prevents dacarbazine from inhibiting cell growth.

Thymidine has earlier been reported to modulate in a synergistic fashion the activity of antineoplastic agents as 5-fluorouracil, arabinofuranosylcytosine, methotrexate, and nitrosoureas (20). It is believed that thymidine influences the cytotoxic agents via the control of the DNA synthetic system although the precise mechanisms are not yet resolved.

The dacarbazine-induced DNA lesions detected in the presence of thymidine appear in replicating DNA. This is inferred by the ability of aphidicolin to prevent the appearance of the DNA lesions. Aphidicolin inhibits DNA polymerases α and δ (18, 19) and therefore stops DNA synthesis. These lesions therefore have the same properties as those described in ref. 2 and are formed either by the incorporation of the drug into DNA or by the attack of the drug on transiently appearing single-stranded DNA during DNA synthesis.

Dacarbazine is a purine analogue. One would therefore expect that exogenous purines should quench the effect of dacarbazine either by preventing the uptake of dacarbazine into the cell or by diluting out the drug intracellularly. However the finding that one pyrimidine but not the other one (thymidine) prevents the appearance of DNA damage indicates that dacarbazine follows specific pathways and that the pathway used by dacarbazine is not connected with the metabolism of thymidine.

One possibility to explain this difference between thymidine and the other three deoxyribonucleosides is their ability to be phosphorylated. Thymidine is phosphorylated by a separate enzyme whereas the same enzyme phosphorylates the other deoxyribonucleosides (11).

Another possible explanation is that the catabolism of thymidine and deoxycytidine differs markedly influencing the ability of the exogenously added substance to compete with dacarbazine.

REFERENCES

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