5-Fluoropyrimidine-induced DNA Damage in Human Colon Adenocarcinoma and Its Augmentation by the Nucleoside Transport Inhibitor Dipyridamole

Ulf Lönn, Sigrid Lönn, Urban Nylen, and Gerard Winblad

ABSTRACT

5-Fluorouracil and 5-fluorodeoxyuridine induce DNA lesions via two different mechanisms, one involving and the other not involving the incorporation of drug into DNA. The DNA lesions are detected by lysing cells in dilute alkali and then separating the DNA in agarose gel electrophoresis. We examine here the effect of dipyridamole, a nucleoside transport inhibitor, on the DNA lesions. We find that dipyridamole augments the levels of DNA fragmentation when the lesions are induced by the mechanism not involving the incorporation of drug. In parallel cytotoxicity is increased.

INTRODUCTION

5-Fluoropyrimidines induce DNA lesions via two different mechanisms, one involving the incorporation of drug into DNA and a second mechanism not involving the incorporation (1). It has been postulated that in the second mechanism the DNA strand breaks detected are in fact "spontaneous" DNA lesions, which are not repaired due to a shortage of dTTP and purines. A similar mechanism has been proposed for the induction of DNA lesions by methotrexate (2) and hydroxyurea (3).

The "spontaneous" lesions appear, e.g., due to misincorporation of uridine during DNA synthesis; deamination of cytosine, adenine, or guanine; or loss of bases resulting in apurinic/apyrimidinic sites. The number of lesions that appear per day in one human cell has roughly been estimated as 10,000 purines and several hundred pyrimidines (4).

Dipyridamole is a well characterized inhibitor of nucleoside transport, not affecting the transport of nucleobases (5). Dipyridamole can therefore be used to enhance the cytotoxicity of antineoplastic agents that inhibit de novo nucleotide synthesis by preventing nucleoside salvage and the restoration of nucleotide pools.

The cytotoxicity of 5-fluoropyrimidines in many cell lines has been correlated with depletion of dTTP pools secondary to inhibition of thymidylate synthase (6, 7). Recently it has been shown that when dipyridamole alters the metabolism of 5-fluorouracil, one can detect a selective increase in 5-fluorodeoxyuridine monophosphate levels (8), which should further augment the inhibitory effect on thymidylate synthase.

In this paper we have examined the presence of DNA lesions in cells treated with 5-fluoropyrimidines (5-FUra3 and 5-FdUrd) and dipyridamole. We show an increase in the level of DNA lesions induced by the 5-fluoropyrimidines without the incorporation of drug into DNA. The result indicates the importance of this mechanism of induction of DNA lesions.

MATERIALS AND METHODS

Cells, Culture Methods, Cytotoxicity Analysis, Labeling with [3H]-Thymidine, and HPLC Analysis. Human colon adenocarcinoma cells (WiDr), obtained from American Type Culture Collection, Bethesda, MD, were grown as described earlier (9). Survival of drug-treated cells was determined by the outgrowth method described in Ref 3. Portions of treated and untreated cells were incubated for 5 days with daily changes of medium. The level of cell survival in treated cultures was measured by determining the difference in the numbers of cell doublings in untreated and treated cell cultures.

To obtain cells with prelabeled DNA, the following incubations were performed: 10^6 cells were seeded in a small culture dish (35 x 10 mm) containing 3 ml of medium with added [3H]thymidine (30 μCi; 24 Ci/mmol; Amersham Inc.). After 24 h the medium was replaced with fresh medium without thymidine, and after another 24 h the cells were used for drug treatment.

HPLC analysis (Spectra-Physics) of 5-fluoropyrimidine metabolites were performed as described (8, 10).

Cell Lysis. The incubation medium was drained off from the culture dish, and the cells were rinsed twice with cold phosphate-buffered saline. Cell lysis was performed in the dark at 0°C by the addition of 2.25 ml of 0.03 M NaOH. After 30 min the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH2PO4. Finally the solution was made 1% with regard to sodium dodecyl sulfate (1, 11).

Agarose Gel Electrophoresis. Agarose flat bed gels (0.75%) were made as described earlier (1). The electrophoretic separation of the DNA was performed using an LKB Multiphor electrophoretic system. After the separation was terminated the gels were sliced into 1-mm thick slices. The slices were incubated in scintillation fluid containing 3% Soluene 100 (Packard), and finally the radioactivity was measured in a scintillation counter (Packard).

RESULTS

It has been reported that increased levels of FdUMP appear when HCT 116 cells (human colorectal carcinoma) are incubated with fluorouracil and the nucleoside transport inhibitor dipyridamole (8). Control experiments with HPLC were performed to examine whether the increase in FdUMP levels reported in HCT 116 cells also exists in WiDr cells (human colon adenocarcinoma). Our HPLC analysis confirms that this is also true in WiDr cells. Therefore we started to examine the DNA of treated cells.

To examine the DNA for the presence of DNA lesions we use a procedure to lyse cells in dilute alkali. If the DNA contains drug-induced lesions, this treatment induces a partial DNA fragmentation whereas DNA from untreated cells appear as high molecular weight DNA (1, 11).

During the lysis in dilute alkali the DNA starts to uncoil, the uncoiling being initiated at gaps present in the DNA due to either the incorporation of drug or the repair of lesions present in the DNA. Under the conditions we use for cell lysis (0.03 NaOH, 0°C, 30 min) there is uncoiling of stretches of DNA amounting to 20 kilobases from each initiation point.

The DNA fragments shorter than 20 kilobases, which are released into solution, remain as single-stranded DNA whereas the high molecular weight DNA renatures to double-stranded

Received 7/11/88; revised 10/13/88; accepted 10/20/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the Stockholm Cancer Society and Karolinska Institute.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: 5-FUra, 5-fluorouracil; 5-FdUrd, 5-fluorodeoxyuridine; FdUMP, fluorodeoxyuridine monophosphate; HPLC, high-performance liquid chromatography.
DNA when the solution is neutralized finally. The fragments are then separated from the high molecular weight DNA by agarose gel electrophoresis.

Fig. 1 shows gel electrophoretic separations depicting the two mechanisms by which 5-fluoropyrimidines induce DNA lesions: Cells with prelabeled DNA are treated with 5-FUra (0.1 mM) for 60 min, which induces DNA fragmentation. However, the fragmentation does not exist when aphidicolin is added to the medium prior to the 5-FUra. Aphidicolin is an inhibitor of DNA polymerase α (12) and prevents the incorporation of drug into DNA by stopping DNA synthesis.

The second mechanism does not involve the incorporation of drug into DNA. Fig. 1B shows that when cells in medium supplemented with aphidicolin are treated with 5-FUra for 6 h one can detect DNA fragmentation. This mechanism does not involve the incorporation of drug into DNA and is the major mechanism used by 5-FdUrd in these cells.

Incubations with Dipyridamole and 5-FUra. Cells with prelabeled DNA were incubated for 6 h with 5-FUra (0.1 mM) (○) or 5-FUra for 30 min and then 5-FUra and dipyridamole (5 μM) for 6 h (●). Cells incubated for 6 h with dipyridamole only (×). The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. (B) Cells treated for 12 h with 5-FUra (○) or 5-FUra for 30 min and then 5-FUra and dipyridamole for 12 h (●).

Fig. 2 shows that in cells treated with dipyrimidamole and 5-FUra there is much higher level of DNA fragmentation than in cells treated with 5-FUra only. The same trend was seen at all time points examined.

In the next set of experiments we analyzed cells preincubated with aphidicolin. Aphidicolin should prevent the increase in DNA fragmentation if it is due to greater incorporation of 5-
Fig. 3. Treatment with aphidicolin. (A) Cells with prelabeled DNA were treated first for 30 min with aphidicolin (10 μg/ml) and then 5-FUra (0.1 mM) and aphidicolin for 6 h (○) or aphidicolin for 30 min, aphidicolin and 5-FUra for 30 min and finally aphidicolin, 5-FUra, and dipyridamole (5 μM) for 6 h (●). The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. (B) The same protocol as in B but the last incubations in medium supplemented with 5-FUra were carried out for 12 h.

Fig. 4. Treatment with dipyridamole and 5-FdUrd. (A) Cells with prelabeled DNA were treated for 6 h with 5-FdUrd (0.1 mM) (○) or 5-FdUrd for 6 h and then 5-FdUrd and dipyridamole (5 μM) for 6 h (●). (B) Treatment for 12 h with 5-FdUrd (○) or 5-FdUrd for 30 min and then 5-FdUrd and dipyridamole for 12 h (●).

FUra into DNA. Fig. 3 shows this not to be the case, implying that it is the mechanism not involving the incorporation of drug into DNA that is modulated.

Incubations with Dipyridamole and 5-FdUrd. 5-FdUrd is not incorporated into the DNA of these cells (1). This drug induces DNA lesions by the mechanism not involving incorporation. We therefore analyzed cells incubated with 5-FdUrd (0.1 mM) for 30 min and then 5-FdUrd and dipyridamole (5 μM) for 6 or 12 h. As controls we incubated cells with 5-FdUrd only.

Fig. 4 shows that in cells treated with dipyridamole and 5-FdUrd there is a higher level of DNA fragmentation than in cells treated with 5-FdUrd only. The same trend is seen at all time points examined.

Furthermore aphidicolin does not alter the levels of DNA fragmentation, in agreement with the interpretation that dipyridamole modulates the mechanism not involving the incorporation of drug (not shown).

Incubations with Dipyridamole and Methotrexate. Methotrexate induces a progressive formation of DNA lesions although the drug is not incorporated into DNA (1, 2). This mechanism corresponds to the second mechanism described for 5-fluoropyrimidines which does not involve the incorporation (1). It has also been reported that dipyridamole augments methotrexate cytotoxicity (13). Therefore we tested the effect on DNA of the combined treatment with methotrexate and dipyridamole.

Cells were incubated with methotrexate (10 μM) for 30 min and then methotrexate and dipyridamole (5 μM) for 6 or 12 h. As controls cells were incubated with methotrexate only. Fig. 5 shows that also in these experiments there are increasing levels...
5-FLUOROPYRIMIDINE-INDUCED DNA LESIONS

Fig. 5. Treatment with dipyridamole and methotrexate. (A) Cells with prelabeled DNA were incubated for 6 h with methotrexate (10 μM) (O) or methotrexate for 30 min and then methotrexate and dipyridamole (5 μM) for 6 h (■). The same protocol as in A but the last incubations with methotrexate were carried out for 12 h.

Cytotoxicity Analysis. To examine cytotoxicity we used an outgrowth method (2). Fig. 6 shows cells incubated for 6 h with 5-FUra, 5-FUra/dipyridamole, 5-FdUrd, or 5-FdUrd/dipyridamole. The data show that cytotoxicity is higher when the 5-fluoropyrimidines are incubated with dipyridamole. Dipyridamole alone has no effect on the cells.

DISCUSSION

In this paper we show that the ability of 5-fluoropyrimidines to induce DNA lesions is augmented by dipyridamole. The levels of DNA lesions are increased using higher concentrations of the 5-fluoropyrimidine or dipyridamole (not shown). It has earlier been reported, and verified by us, that the combined treatment results in increased levels of FdUMP as compared to cells treated with 5-fluoropyrimidines only (8).

The high level of FdUMP would augment the inhibitory effect of the drug on thymidylate synthase. The now more efficient inhibition of the enzyme (as compared to cells treated with 5-fluoropyrimidines only) would further reduce the production of new nucleotides.

Furthermore pyridamole inhibits the uptake of nucleosides from the medium and thereby also reduces salvage pathways for the production of nucleotides. Taken together this means that in the treated cells there exists a shortage of nucleotides. It is known that dTTP pools are reduced to one-half of control values by treatment with 5-FUra and that the pools are further depleted by the addition of dipyridamole (8, 14).

We report here that the combined action of 5-fluoropyrimidines and dipyridamole induces very high levels of DNA lesions. The effects are similar for both 5-FUra and 5-FdUrd and are paralleled by increased cytotoxicity.

There are two known mechanisms by which 5-fluoropyrimidines induce DNA lesions (1). The first mechanism involves...
the incorporation of drug into DNA whereas the second mechanism does not. Aphidicolin inactivates the first mechanism by stopping DNA synthesis.

However, aphidicolin does not prevent the increase in the levels of DNA lesions in cells treated with 5-fluoropyrimidines and dipyrimidamole. It therefore seems very likely that the lesions appear due to the second mechanism, the one not involving the incorporation of drug into DNA. Further support for this is seen in the data showing that dipyridamole also increases the level of DNA lesions induced by methotrexate. Methotrexate induces DNA lesions by a mechanism similar to the second mechanism of 5-fluoropyrimidines (1, 2).

The data presented here underline the importance of the second mechanism to induce DNA lesions. By increasing its efficiency it should be possible to gain more efficient cell killing in the therapeutic setting.

REFERENCES

5-Fluoropyrimidine-induced DNA Damage in Human Colon Adenocarcinoma and Its Augmentation by the Nucleoside Transport Inhibitor Dipyridamole

Ulf Lönn, Sigrid Lönn, Urban Nylen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/5/1085

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.