Interaction between 5-Fluorouracil and DNA of Human Colon Adenocarcinoma

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ABSTRACT

The effect of 5-fluorouracil on the stability of DNA and the synthesis of DNA replication intermediates was analyzed in human colon adenocarcinoma cells. Density gradient analysis showed that some of the drug is incorporated into DNA. Moreover, DNA fragments are released when cells with drug-containing DNA are lysed in dilute alkali. The DNA fragments can be separated from the bulk DNA by agarose gel electrophoresis. The fragmentation of the DNA can be prevented by pretreatment with aphidicolin which inhibits DNA polymerase α.

In 5-fluorouracil-treated cells, a heterogeneous population of DNA replication intermediates is formed, instead of discrete DNA replication intermediates which are formed in untreated cells. Aphidicolin prevents the formation of the heterogeneous population of DNA fragments. However, replication intermediates formed before the blockade with aphidicolin are ligated to high-molecular-weight DNA. In cells released from aphidicolin inhibition, there is preferential labeling of the heterogeneous population of DNA fragments. This population, therefore, shows the same characteristics as the discrete DNA populations formed in untreated cells.

INTRODUCTION

Survival after surgical removal of colonic carcinomas has not changed during the last 30 years, due mainly to our inability to treat disseminated disease. The introduction of the fluorinated pyrimidines in 1958 was followed by intense clinical investigations to develop effective systemic therapy for disseminated colon cancer. However, thus far, 5-FUra³ and its analogues are the only drugs that show a reproducible but modest therapeutic effect with response rates on the order of 20%. Thus far, it has not been possible to combine 5-FUra with other drugs to obtain combination chemotherapy programs with higher response rates. So far, 5-FUra and its analogues have not been able to prevent the development of multidrug resistance and to improve the survival after surgical removal of colon cancer.

Recently, it has been shown that 5-FUra is incorporated into the DNA of human breast cancer cells (14) and the DNA of HeLa cells (5). The significance of this incorporation is thus far not completely understood. Our knowledge of the chromosome structure and the synthesis of DNA has increased during the last years. It is, e.g., now possible to detect different well-defined DNA replication intermediates and more accurately than before to examine the influence of different DNA polymerases on DNA replication or DNA repair.

To detect DNA replication intermediates, one can use a procedure of cell lysis in dilute alkali, followed by gel electrophoretic separation of the DNA (13). The same approach can be used to analyze if drugs induce lesions in the DNA, since many lesions result in alkali-sensitive DNA.

In order to gain further insight into the interaction between 5-FUra and DNA of colon carcinomas, we have used this technique asking the question: whether 5-FUra is incorporated into the DNA and if such incorporation affects the synthesis of DNA replication intermediates. We have found that in the cell line that we analyze, incorporation of 5-FUra into DNA occurs at a low but significant level. The incorporation can be prevented by inhibiting DNA polymerase α. Also formation of DNA replication intermediates is changed in treated cells.

MATERIALS AND METHODS

Cells, Culture Methods, and Labeling with [³H]Thymidine. Human colon adenocarcinoma cells (WiDr), obtained from American Type Culture Collection, Bethesda, MD, were grown as monolayers in 75-sq cm plastic flasks (Falcon Plastics) at 37° in a humidified atmosphere of 5% CO₂ in air. The culture medium was Eagle's minimal essential medium with Earle's salts, containing 2 mm l-glutamine, 10% heat-inactivated fetal calf serum, and antibiotics. The culture medium was routinely changed twice weekly, and the cells were passaged every 4 to 6 days.

For experiments involving steady state labeled DNA, the cells were seeded in small culture dishes (35 x 10 mm) containing 3 ml medium. Tritiated thymidine (30 μCi; 20 Ci/mmol; Amersham, Inc.) was added to the culture medium. After 24 hr, the medium was changed to fresh medium without thymidine; after another 24 hr, the cells were used for drug treatment experiments. For pulse-labeling experiments, 75 μCi tritiated thymidine were added to the medium for the appropriate period of time.

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° by the addition of 2.25 ml of 0.03 M NaOH. After 30 min, the solution was neutralized by addition of 0.9 ml of 0.067 M HCl 0.02 M NaH₂PO₄. For a more detailed description of the cell lysis procedure, see Ref. 12. Finally, the solution was made 1% with regard to sodium dodecyl sulfate.

Agarose Gel Electrophoresis and Cs₂SO₄ Gradient Centrifugation. Agarose flat-bed gels (0.75%) were made as described earlier (11). The electrophoretic separation of the DNA was performed using an LKB Multiphor electrophoretic system. After the separation was terminated, the gels were sliced in 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). For Cs₂SO₄ gradient centrifugations, the DNA was dissolved in 0.002 M Tris-HCl (pH 8)-0.001 M disodium EDTA, and Cs₂SO₄ was added to a refractive index of 1.375. Centrifugation was carried out at 40,000 rpm at 20° for 48 hr in a Ti 50 rotor using a Beckman ultracentrifuge. After

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³ The abbreviation used is: 5-FUra, 5-fluorouracil.

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RESULTS

Incorporation of 5-FUra into Both RNA and DNA of Human Colon Adenocarcinoma Cells. 5-FUra is known to rapidly inhibit the activity of the enzyme thymidylate synthetase. The effect on DNA synthesis sets in gradually, in all probability as a result of a developing shortage of nucleotides. In tumor-bearing mice treated with 5-FUra, DNA synthesis continues at a low rate for 12 hr and then ceases completely (15). When we examined the incorporation into cold trichloroacetic acid-precipitable material of tritiated thymidine administered as a 5-min pulse after treatment of the cells with 5-FUra for 60 min, we found that the incorporation was 77% of that in untreated control cells.

5-FUra is a pyrimidine analogue and should theoretically function as a precursor for both RNA and DNA. It is well established that tritiated 5-FUra is incorporated into RNA. In order to examine whether 5-FUra is incorporated into DNA as well, we have carried out neutral CsSO4 gradient centrifugations. For these experiments, we have patterned the labeling protocol used by Major et al. (14), who demonstrated incorporation of 5-FUra into the DNA of human breast cancer cells.

The CsSO4 gradient analysis shows that the majority of the label is detected in RNA. However, there is also label in DNA, although there is less of it than in the RNA band (Chart 1). If the sample has been treated with DNase before centrifugation, the label in the DNA band disappears. We conclude that in colon adenocarcinoma cells 5-FUra is incorporated into both RNA and DNA.

Induction of Fragmentation of Steady State Labeled DNA by 5-FUra. In order to characterize cells treated with 5-FUra, we examined the effect of 5-FUra on steady state labeled DNA. It is known that many drugs induce alkali-labile bonds in DNA, either because of the incorporation of the drug into the DNA or during the repair of lesions induced in the DNA.

To detect alkali-labile bonds in the DNA of 5-FUra-treated cells, we lyse the cells in dilute alkali which results in denaturation of the DNA. However, the DNA strands cannot separate before enough time has elapsed to allow unwinding of the DNA strands [see Ahnström and Erixon (1)]. The unwinding of the DNA is initiated at gaps present in the DNA, e.g., due to the presence of alkali-labile bonds. The stretch of DNA that can unwind at each side of a gap in the alkaline medium (0.03 M NaOH, 0°C) has been estimated to about 2 × 10⁶ daltons (6).

When the alkaline solution is neutralized, the high-molecular-weight DNA renatures and forms double-stranded DNA, whereas small DNA fragments, formed as a result of DNA helix unwinding at alkali-labile bonds, remain free in the solution as single-stranded DNA. When this mixture of DNA molecules is examined by agarose gel electrophoresis, it is possible to separate the high-molecular-weight DNA from smaller DNA fragments and thus detect whether the cells contain DNA with alkali-labile bonds.

Cells with steady state labeled DNA were treated with 1 mm 5-FUra for 60 min and then lysed in dilute alkali. Electrophoretic separation of the sample shows a partial fragmentation of the DNA (Chart 2A). Apart from the high-molecular-weight DNA, one can also detect a population of labeled DNA fragments at Slices 20 to 30. This population of DNA fragments is absent in cells not treated with 5-FUra. Hence, cells treated with 5-FUra contain DNA with alkali-labile bonds.

The results of CsSO4 gradient centrifugations described above indicate that 5-FUra is incorporated into the DNA. A possible explanation for the induction of alkali-labile bonds in the DNA is that 5-FUra is incorporated into the DNA during semiconservative DNA synthesis, since 5-FUra is a pyrimidine analogue.

Pretreatment with Aphidicolin Prevents 5-FUra from Fragmenting the DNA. Aphidicolin is a specific inhibitor of DNA polymerase α, thereby inhibiting the movement of the replication fork (9). We have used this drug to examine whether there is a correlation between the movement of the replication fork and the ability of 5-FUra to induce damage in the DNA.

Cells with steady state labeled DNA were treated with aphidicolin for 60 min and then with 5-FUra plus aphidicolin for another 60 min. We have shown earlier that a treatment with aphidicolin for 60 min stops the movement of the replication forks (13). This experimental protocol, as shown in Chart 2B, prevents 5-FUra from inducing damage in the DNA, indicating that 5-FUra induces damage in the DNA during semiconservative DNA synthesis.

Formation of DNA Replication Intermediates in 5-FUra-treated Cells. 5-FUra is incorporated into the DNA. It is possible that the presence of 5-FUra in the replication intermediates changes the formation and/or processing of the intermediates. A change in size distribution of intermediates may also occur.

![Chart 1. Banding of DNA labeled with 5-[3H]FUra in neutral CsSO4 gradients. The cells were incubated according to the protocol of Major et al. (14), i.e., incubated in serum-free medium with 1 μM tritiated 5-FUra (12 Ci/mmol; Amersham Inc.) for 12 hr. The cells were lysed at neutral pH. The nucleic acids were purified and then analyzed by CsSO4 gradient centrifugation. Inset scale, amount of CsSO4 in g/ml in the different fractions.](image-url)
Chart 2. In A, cells with steady state labeled DNA were treated with 5-FUra (1 μM) for 60 min (○). Untreated cells (□) and the 5-FUra-treated cells were lysed in dilute alkali, and the labeled DNA was then separated by electrophoresis in 0.75% agarose gels. 25, 10 and 2, size (in kilobases) and location of single-stranded DNA markers. In B, cells with steady state labeled DNA were treated with aphidicolin (10 μg/ml) (□) for 60 min before the addition of 5-FUra (1 μM). The subsequent treatment with aphidicolin and 5-FUra was performed for 60 min. Control cells received treatment with 5-FUra only (○). After cell lysis, the labeled DNA was analyzed in 0.75% agarose gels.

Chart 3. In A, cells were incubated with 5-FUra (1 μM) for 60 min and either pulsed with tritiated thymidine during the last 5 min of the treatment (○) or incubated in fresh medium for 60 min before pulse-labeling for 5 min (□). Another group of cells was treated simultaneously with 5-FUra and aphidicolin (10 μg/ml) for 60 min and pulsed with tritiated thymidine for the last 5 min of the treatment (△). The cells were lysed in dilute alkali, and the DNA was then analyzed in 0.75% agarose gels. The 10-kilobase DNA replication intermediates are located at Slices 19 to 25, and the Okazaki fragments are located at Slices 37 to 45. 25, 10, and 2, size (in kilobases) and location of single-stranded DNA markers. In B, cells were incubated with 5-FUra for 60 min and pulsed with tritiated thymidine during the last 5 min (□) and then incubated for 30 min in the presence of both 5-FUra and aphidicolin (△). After cell lysis, the labeled DNA was analyzed in 0.75% agarose gels. In C, cells were incubated simultaneously with 5-FUra and aphidicolin for 60 min, washed free of the drugs, and then pulsed with tritiated thymidine for 5 min (□) or 15 min (△). After cell lysis, the labeled DNA was analyzed in 0.75% agarose gels.

To examine this question, we treated cells with 5-FUra for 60 min and labeled them with tritiated thymidine during the last 5 min of the drug treatment. We have earlier established that in untreated cells there exist 2 discrete populations of DNA replication intermediates, 10 kb DNA and Okazaki fragments (13). However, in 5-FUra-treated cells, one cannot detect discrete replication intermediates. Instead, the electrophoretic separation shows a heterogeneous population of molecules covering the size from 10 kilobases down to Okazaki fragments (Chart 3A). Furthermore, the distribution of molecules is asymmetrical, with more labeled material in the low-molecular-weight region.

In contrast, if the cells have been washed free of 5-FUra, incubated in fresh medium for 60 min, and then pulsed with tritiated thymidine, discrete DNA replication intermediates, the 10-kilobase DNA at Slices 19 to 25, and Okazaki fragments at Slices 37 to 45 can be detected. This is the same picture as described earlier for untreated cells (13). Hence, the effect of 5-FUra on the size distribution of the DNA replication intermediates is reversible.

Experiments using aphidicolin were performed to ensure that the heterogeneous population of molecules covering the size from 10 kilobases down to Okazaki fragments are true replication intermediates. Aphidicolin is a specific inhibitor of DNA polymerase α, the polymerase responsible for chromosomal DNA synthesis (9). Cells were incubated simultaneously with 5-FUra and aphidicolin and labeled with tritiated thymidine during the last 5 min of the drug treatment. As seen in Chart 3A, the presence of aphidicolin abolishes the labeling of the heterogeneous material.
Ligation of the Heterogeneous DNA Replication Intermediates Occurs in the Presence of Aphidicolin. There is some incorporation of label into high-molecular-weight DNA in cells treated with both aphidicolin and 5-FUra (Chart 3A). The results resemble the findings that we have obtained with cells treated with aphidicolin but no 5-FUra (13). The incorporation of label into high-molecular-weight DNA in the presence of aphidicolin occurs because of continued ligation of large replication intermediates (10-kilobase DNA intermediates), although there is no formation of new intermediates since the movement of the replication forks is stopped.

To examine whether the heterogeneous material behaves as the 10-kilobase DNA replication intermediates, the following experiments were performed. Cells were incubated with 5-FUra for 60 min and pulsed with tritiated thymidine during the last 5 min of the drug treatment. Aphidicolin was then added, and the cells were incubated for another 30 min. Chart 3B shows that in cells treated with aphidicolin there is an increase in labeled high-molecular-weight DNA and a parallel reduction in label in the heterogeneous DNA replication intermediates. However, one can still detect Okazaki fragments. The results indicate that there is ligation of the heterogeneous DNA replication intermediates in the presence of aphidicolin.

In cells treated with aphidicolin and 5-FUra before the pulse with tritiated thymidine, one would expect aphidicolin to cause an accumulation of nonlabeled Okazaki fragments and a lack of the heterogeneous DNA replication intermediates. When aphidicolin is removed, one would therefore expect that pulse labeling of the cells should preferentially detect the intermediates larger than the Okazaki fragments, due to the incorporation of label when the nonlabeled Okazaki fragments are joined to larger intermediates. This situation occurs in cells not treated with 5-FUra (13). Chart 3C shows that immediately after the removal of the drugs one can detect radioactivity in the heterogeneous DNA population but only little of it in Okazaki fragments. However, after a longer pulse with tritiated thymidine, one can also visualize labeled Okazaki fragments and also a small amount of 10-kilobase DNA superimposed on the heterogeneous DNA population.

**DISCUSSION**

Recently, it has been shown that 5-FUra is incorporated into the DNA of human breast cancer cells (14) and HeLa cells (5). In most cells thus far examined, it has been difficult to detect incorporation of 5-FUra into DNA, probably due to efficient dUTP hydrolases or uracil-DNA glycosylases (4, 10). In this paper, we have analyzed the interaction between 5-FUra and the DNA of a human colon adenocarcinoma cell line.

5-FUra is the major drug used in the treatment of disseminated colon cancer. However, thus far, it has not been possible to construct efficient chemotherapeutic regimes in this disease, probably because of inadequate knowledge of the mechanisms by which 5-FUra exerts its effects. Therefore, we wished to analyze whether 5-FUra is incorporated into the DNA of colon cells and if so which effect such an incorporation has on the stability of the DNA and the formation of DNA replication intermediates.

To establish whether tritiated 5-FUra is incorporated into the DNA, we have used gradient centrifugations in Cs2SO4. The results show that although the major part of the label is incorporated into RNA there is a distinct peak of labeled DNA.

Next, we wished to analyze whether there is a change in the stability of steady state labeled DNA. In order to test whether 5-FUra induces alkali-labile sites in the DNA, we have used a technique of lysing the cells in dilute alkali. After neutralization of the solution, the labeled DNA is separated by agarose gel electrophoresis. In untreated cells, one can detect a labeled peak of high-molecular-weight DNA. However, in 5-FUra-treated cells, it is possible to detect apart from the high-molecular-weight DNA, also a peak of smaller DNA fragments, indicating that the incorporation of 5-FUra in the DNA induces alkali-labile sites which results in fragmentation of the DNA.

We have examined whether the incorporation of 5-FUra in the DNA is due to semiconservative DNA synthesis, by inhibiting DNA polymerase α, the enzyme responsible for semiconservative DNA synthesis (9), with the drug aphidicolin. When cells pretreated with aphidicolin are treated with 5-FUra, we do not find any fragmentation of the steady state labeled DNA. This strongly indicates that the incorporation of 5-FUra in DNA occurs during semiconservative DNA synthesis.

Using our procedure of cell lysis in dilute alkali, one can detect 2 discrete populations of DNA replication intermediates in untreated cells (13). In cells treated with 5-FUra, we see a change in size distribution of replication intermediates. Instead of 2 discrete populations, 10-kilobase DNA, and Okazaki fragments, one can detect a heterogeneous population of molecules ranging in size from 10 kilobases down to Okazaki fragments.

The heterogeneous population of molecules exhibits the same properties as do large DNA replication intermediates (10-kilobase DNA) detected in cells not treated with 5-FUra. The intermediates are not formed in cells pretreated with aphidicolin in which DNA polymerase α is inhibited. However, the intermediates formed before the addition of aphidicolin are ligated to high-molecular-weight DNA, whereas already formed Okazaki fragments accumulate in the cell. Moreover, in cells released from aphidicolin block, there is preferential labeling of the heterogeneous DNA population.

One likely explanation of the findings is that nicking or gap formation is going on extensively in the intermediates, through a series of reactions involving uracil-DNA glycosylases to remove incorporated 5-FUra. Such gap formation should result in fragmentation of 10-kilobase DNA when cells are lysed in dilute alkali, resulting in a heterogeneous population of DNA molecules. This interpretation is supported by the finding that when the cells have been washed free of 5-FUra the formation of discrete 10-kilobase DNA replication intermediates resumes.

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