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THE INCREASED CYTOTOXICITY IN COLON ADENOCARCINOMA OF METHOTREXATE–5-FLUOROURACIL IS NOT ASSOCIATED WITH INCREASED INDUCTION OF LESIONS IN DNA BY 5-FLUOROURACIL

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Abstract—Sequential methotrexate–5-fluorouracil (Mtx–5-Fu) reduces proliferation of human colon adenocarcinoma cells. The DNA of treated cells was examined by cell lysis in dilute alkali in order to detect if there is any release of single-stranded DNA fragments, which occurs in cells treated with 5-Fu. The results showed that in spite of increased cytotoxicity of Mtx–5-Fu there is reduced release of DNA fragments. The findings are paralleled by a reduced incorporation of 5-Fu into DNA.

Hypoxanthine reduces the growth inhibitory effect of Mtx–5-Fu. In treated cells grown in the presence of hypoxanthine, alkaline lysis causes release of DNA fragments from bulk DNA, in contrast to cells grown without hypoxanthine.

Hence, the increased cytotoxicity of Mtx–5-Fu in human colon adenocarcinoma is not associated with enhanced lesions in DNA by 5-Fu.

Methotrexate (Mtx), a folic acid antagonist, and 5-fluorouracil (5-Fu), a pyrimidine analogue, are powerful cytotoxic agents used in the clinical treatment of neoplasms [1]. Combination chemotherapy regimens containing Mtx and 5-Fu have been used for many years, particularly in the treatment of disseminated breast cancer. Furthermore, during the last few years a synergistic effect of the sequential treatment of Mtx–5-Fu has been reported. Clinical studies have been done primarily on breast, colon and squamous cell head and neck cancers.

The biochemical basis for the increased cytotoxic action of sequential Mtx–5-Fu is not completely understood. Several proposals have been put foward to explain the synergistic effect, e.g. enhanced binding of 5-fluorodeoxyuridine (a metabolite of 5-Fu) to the enzyme thymidylate synthethase in the presence of increased levels of dihydropteroylpolyglutamates. Another proposed mechanism is increased incorporation of 5-Fu into RNA due to enhanced drug metabolism [1–7].

We have earlier examined the interaction between 5-Fu and the DNA of human colon adenocarcinoma [8]. In agreement with some recent reports from other cells we have shown that 5-Fu is incorporated into the DNA [5, 6, 9, 10]. The significance of the induction of lesions in the DNA with regard to the cytotoxic effect of sequential Mtx–5-Fu is not understood so far.

The experimental approach we use reveals that DNA containing 5-Fu is sensitive to alkali. Cell lysis in dilute alkali results in the detection of single-stranded DNA fragments, as well as high molecular weight DNA [8]. In untreated cells one can detect only high molecular weight DNA [11].

Furthermore, DNA replication intermediates which contain 5-Fu show a different size spectrum than DNA replication intermediates from untreated cells. In untreated cells it is possible to detect a discrete 10 kb DNA replication intermediate, which is not formed in treated cells [8, 11].

Our experimental approach allows the examination of lesions induced in the DNA by 5-Fu. We have now examined the effect of treatment with sequential Mtx–5-Fu on DNA of human colon adenocarcinoma cells. The results show that with increased cytotoxicity fewer lesions are induced in the DNA by 5-Fu.

MATERIALS AND METHODS

Cells, culture methods and labelling with [^{3}H]-*thymidine*

Human colon adenocarcinoma cells (WiDr), obtained from American Type Culture Collection, Bethesda, MD, were grown as earlier described [12]. However, in the experiments reported here we have used dialysed fetal calf serum (FCS), obtained by dialysis against Hank's balanced salt solution. The number of cells per culture dish was determined with an Analys Instrument Cell Counter 134.

For experiments we use cells with pre-labelled DNA. For this 10^6 cells were seeded in a small culture dish (35 × 10 mm) containing 3 ml medium with added [^{3}H]thymidine (30 μCi; 20 Ci/mmole; Amersham, Inc.). After 24 hr, the medium was replaced with fresh medium without thymidine; after another 24 hr, the cells were used for drug treatment.

For the $CsSO_4$-gradient analysis, cells were incubated with [^{3}H]5-Fu (12 Ci/mmole; Amersham, Inc.), basically according to the protocol of Major *et al.* [10]. Mtx and 5-Fu were obtained from Sigma Biochemicals.

Cell lysis

The incubation medium was drained off from the culture dish, and the cells rinsed twice in 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 the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH_2PO_4. For a more detailed description of the cell lysis procedure, see [12, 13]. Finally, the solution was made 1% with regard to SDS.

For the $CsSO_4$-gradient analysis we lysed the cells at neutral pH [10].

Agarose gel electrophoresis and $CsSO_4$-gradient centrifugations

Agarose flat-bed gels (0.75%) were made as described earlier [14]. 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).

For $CsSO_4$-gradient centrifugations, the DNA was dissolved in 0.02 M Tris–HCl (pH 8)–0.001 M EDTA, and $CsSO_4$ 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 centrifugation, fractions were collected by siphoning from the bottom of the centrifugation tube. The DNA was precipitated with cold trichloroacetic acid and the radioactivity was measured by scintillation counting (Packard).

RESULTS

Proliferation of human adenocarcinoma cells treated with drugs

We have examined the proliferation of human adenocarcinoma cells incubated with drugs for short periods of time. The incubations with Mtx (10 μM) or 5-Fu (1 mM) were for 60 min. For sequential Mtx–5-Fu the cells were incubated with Mtx (10 μM) for 60 min, followed by 60 min incubation in fresh medium, and finally 60 min incubation in medium with 5-Fu (1 mM).

Figure 1 shows that the treatment with Mtx–5-Fu causes reduced cell proliferation. Cells treated with either 5-Fu or Mtx alone proliferate almost as well as untreated cells.

It has been reported that the presence of purines in FCS may reduce the synergistic action of the sequential treatment with Mtx–5-Fu [16]. In all experiments reported here we have used FCS, which has been dialysed in order to remove purines if present.

We also examined the proliferation of cells incubated in the presence of 30 μM hypoxanthine during the treatment with Mtx–5-Fu. As shown in Fig. 1 this incubation condition does not slow cell proliferation, in contrast to cells incubated with Mtx–5-Fu in the absence of hypoxanthine.

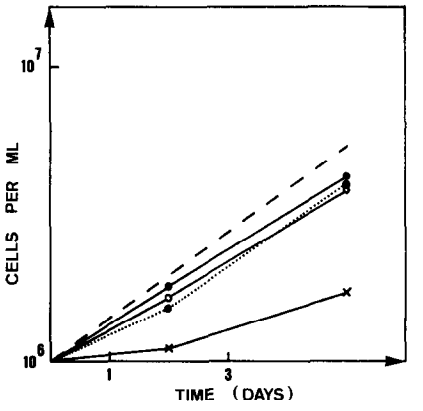

Fig. 1. Cell proliferation of human colon adenocarcinoma cells treated with 5-Fu (1 mM) for 60 min (—○—), Mtx (10 μM) for 60 min (—●—) or sequential Mtx–5-Fu, Mtx (10 μM) for 60 min + 60 min fresh medium + 60 min 5-Fu (1 mM), (—×—). Sequential Mtx–5-Fu in the presence of hypoxanthine (300 μM) (···●···). Untreated cells (---). The cells were allowed to grow in fresh medium for five days. The number of cells were determined using an Analysis Instrument Cell Counter 134.

Stability of DNA in cells treated with methotrexate

In order to analyse the stability of DNA in cells treated with Mtx for 60 min, we use the following approach [8, 11]. Cells are lysed 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 [13]. The unwinding of the DNA is initiated at gaps present in the DNA, e.g. due to the presence of alkali-labile bonds induced during drug-treatment of cells. The stretch of DNA that can unwind at each side of a gap during our alkaline cell lysis procedure (0.03 M NaOH, 0°, 30 min) has been estimated to be about 2×10^7 Daltons [15].

When the alkaline solution is neutralized, the high molecular weight DNA renatures and forms double-stranded DNA. In contrast, small single-stranded DNA fragments formed as a result of the existence of alkali-labile bonds, remain free in the solution. These DNA fragments can be separated from the high molecular weight DNA by agarose gel electrophoresis. We have earlier used this approach to analyse the formation of DNA replication intermediates and the induction of alkali-sensitive regions in DNA by 5-Fu [8, 11].

Cells with steady-state labelled DNA were treated with Mtx (60 min, 10 μM), then lysed in dilute alkali and the DNA analysed in agarose gels. As shown in Fig. 2(A), the treatment with Mtx does not change the electrophoretic profile (only high molecular weight DNA detected) and therefore does not induce alkali-labile bonds in the DNA.

The results contrast with those obtained by a treatment with 5-Fu (60 min, 1 mM), which produces a partial fragmentation of the DNA [8]. Figure 2(A) shows that in 5-Fu treated cells, apart from the high molecular weight DNA, there also exists a population of DNA fragments located at slices 20–30. This population does not exist in either untreated cells nor Mtx-treated cells.

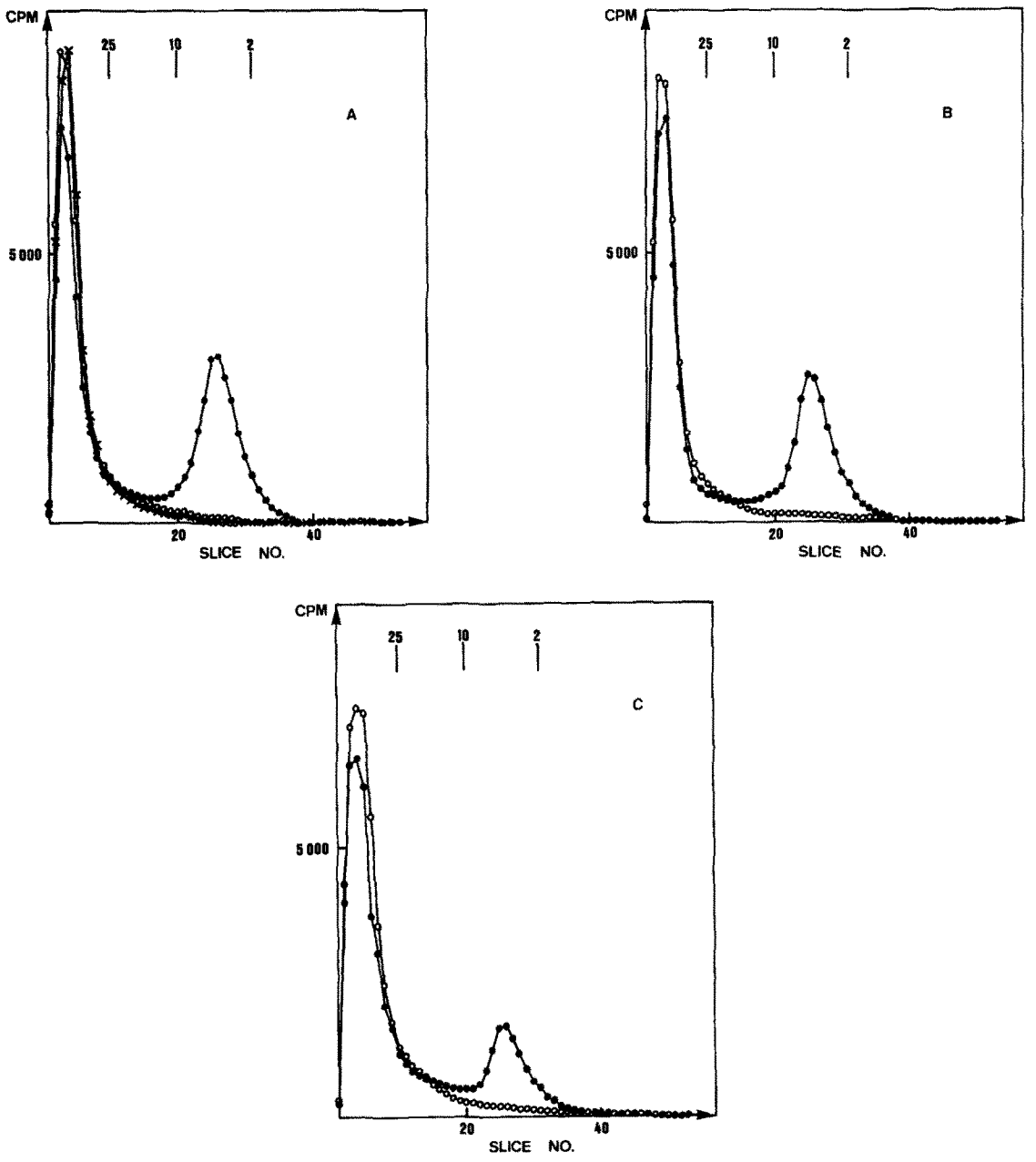

Fig. 2. (A) Colon adenocarcinoma cells with steady-state labelled DNA were treated with 5-Fu (1 mM) for 60 min (—●—), or with Mtx (10 μM) for 60 min (—○—). Untreated cells (—×—). The same number of cells were used in all experiments. The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. Numerals across the top (25, 10 and 2) denote the size (in kb) and location of single-stranded DNA markers. The high molecular weight DNA is located at slice 3–6 and the single-stranded DNA fragments are located at slices 20–30. (B) Cells with steady-state labelled DNA were treated with sequential Mtx–5-Fu, Mtx (10 μM) for 60 min + 60 min in fresh medium + 60 min 5-Fu (1 mM), (—○—), or treated with 5-Fu (1 mM) for 60 min (—●—). (C) Cells with steady-state labelled DNA were treated with sequential Mtx–5-Fu in the presence of hypoxanthine (—●—). Cells incubated in Mtx–5-Fu in the absence of hypoxanthine (—○—).

Pretreatment with methotrexate prevents 5-Fu from inducing damage in the DNA

Cells treated with sequential Mtx–5-Fu proliferate slower than cells treated with 5-Fu. Therefore we wished to examine whether the pretreatment with Mtx influences the ability of 5-Fu to fragment DNA.

Cells with steady-state labelled DNA were treated with Mtx (10 μM) for 60 min, incubated for 60 min in fresh medium and then incubated for 60 min in the presence of 5-Fu (1 mM). Celly lysis was performed in dilute alkali. When the labelled DNA was separated by agarose gel electrophoresis the results did not show any fragmentation of the DNA [Fig. 2(B)].

Hence, a pretreatment with Mtx reduces the ability

of 5-Fu to damage DNA, although the cells proliferate less than cells treated with only 5-Fu.

Addition of hypoxanthine to the medium results in fragmentation of the DNA during methotrexate–5-Fu

We have dialysed FCS in order to use medium with no or very low concentrations of purines. This facilitates the cytotoxic effect of sequential Mtx–5-Fu.

The presence of low levels of hypoxanthine is important for the synergistic cytotoxicity [16]. Therefore we wished to examine whether the addition of hypoxanthine to the culture medium may counteract the protective capacity of Mtx, allowing 5-Fu now to induce fragmentation of the DNA. As was shown in Fig. 1, the addition of hypoxanthine prevents Mtx–5-Fu from reducing the proliferation capability of the cells treated with Mtx–5-Fu.

Cells with steady-state labelled DNA were incubated with hypoxanthine (30 μM) for 3 hr, during which time the cells were also treated according to the following protocol: incubation with Mtx for 60 min, 60 min in medium without Mtx and finally 60 min in medium with 5-Fu.

When the labelled DNA was examined in agarose gels, it was possible to detect fragmentation of the DNA, with labelled material located at slices 20–30. The picture is the same as detected in cells treated with only 5-Fu [Fig. 2(C)]. The fragmentation is not as extensive as in cells treated with 5-Fu only, but it is nevertheless clearly detectable.

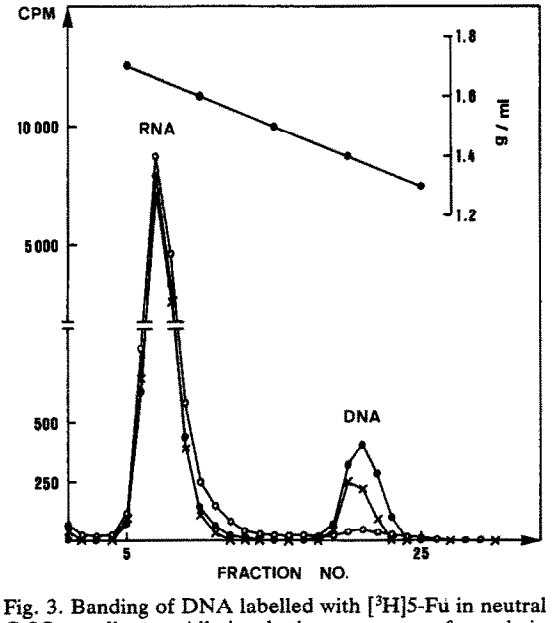

Fig. 3. Banding of DNA labelled with [^{3}H]5-Fu in neutral $CsSO_4$-gradients. All incubations were performed in serum-free medium [10]. The same number of cells were used in all experiments. The cells were lysed at neutral pH, the nucleic acids purified and then analysed by $CsSO_4$-gradient centrifugations [10]. Cells incubated with 1 μM [^{3}H]5-Fu (12 Ci/mmole; Amersham, Inc.) for 60 min (—●—). Sequential Mtx–5-Fu with [^{3}H]5-Fu during the 60 min of 5-Fu treatment (—○—). Sequential Mtx–5-Fu in medium containing hypoxanthine (30 μM); [^{3}H]5-Fu was added during the 60 min of 5-Fu (—×—).

Hence when hypoxanthine is present in the medium during sequential Mtx–5-Fu one can detect fragmentation of DNA.

$CsSO_4$-gradient separations of [^{3}H]5-Fu labelled DNA

The results above show that Mtx modulates the capability of 5-Fu to induce damage in DNA. A possible explanation for this effect is that Mtx influences the incorporation of 5-Fu into DNA. To examine this question we have used $CsSO_4$-gradients.

Banding in $CsSO_4$ allows the distinction between incorporation of [^{3}H]5-Fu into DNA and RNA. Figure 3 shows that in cells treated with [^{3}H]5-Fu there is a small but distinct incorporation into DNA. The incorporation does not, however, occur in cells treated with sequential Mtx–[^{3}H]5-Fu. In contrast, in cells treated with Mtx–[^{3}H]5-Fu in the presence of hypoxanthine, incorporation of 5-Fu into the DNA does occur.

The results agree well with the findings described above. Increased cytotoxicity (sequential Mtx–5-Fu) is accompanied by lower incorporation of 5-Fu into the DNA and less fragmentation of pre-labelled DNA. Conversely, in lower cytotoxicity (sequential Mtx–5-Fu in medium containing hypoxanthine) there is incorporation of 5-Fu into the DNA and fragmentation of pre-labelled DNA.

DISCUSSION

When 5-Fu was introduced into cancer chemotherapy it was believed that the drug was not incorporated into DNA, although 5-Fu is a pyrimidine analogue. Recent findings show that incorporation occurs and that the earlier erroneous results were caused by efficient removal of 5-Fu from DNA by cellular DNA glycolases [4–6, 9, 10]. The experimental approach we use allows the detection of DNA fragments released from 5-Fu containing bulk DNA [8]. We now use the presence or absence of the DNA fragments as a marker for the induction of 5-Fu lesions in the DNA during treatment of cells with sequential Mtx–5-Fu.

Cell proliferation in the human colon adenocarcinoma cell line is strongly reduced by sequential treatment with Mtx–5-Fu. Therefore, we examined whether Mtx influences the ability of 5-Fu to fragment steady-state labelled DNA. We found that the treatment with Mtx before the treatment with 5-Fu prevents the fragmentation of DNA. Nevertheless, the sequential Mtx–5-Fu reduces cell proliferation, indicating that the cytotoxic effect of this combination of drugs is not mediated by increased induction of lesions in the DNA by 5-Fu.

In order for the Mtx–5-Fu combination to show synergistic cytotoxicity, the concentration of purines in the culture medium should be kept as low as possible [16]. Therefore in our experiments we have used dialysed FCS.

Addition of hypoxanthine to the medium reverses the inhibitory effect of the drugs on cell proliferation. Therefore we have examined whether the ability of

Mtx to prevent the 5-Fu induced fragmentation of DNA is changed when hypoxanthine is added to the culture medium. In cells treated in this manner one can detect fragmentation of steady-state labelled DNA similar to that detected in cells incubated with 5-Fu only. Hence when the cytotoxic effect is lowered (hypoxanthine added to the medium) it is possible to detect the induction of 5-Fu induced lesions in the DNA. In contrast, when the sequential Mtx–5-Fu causes high cytotoxicity one cannot detect fragmentation of bulk DNA.

Furthermore experiments using $CsSO_4$-gradient centrifugations showed a large incorporation of 5-Fu into RNA but very little, if any, incorporation into DNA during sequential Mtx–5-Fu (higher cytotoxocity). The gradient results showed, however, resumed incorporation of 5-Fu into DNA during incubation of cells in medium with hypoxanthine (lower cytotoxicity).

In some cell types examined it has been found that incorporation of 5-Fu into DNA is unchanged when cells are pretreated with Mtx [17], where incorporation is increased in other cells [18]. Also in some cells it has been found an increased induction of lesions in DNA with increased cytotoxicity during single-drug treatement with 5-Fu [19]. It is possible that the differences in results reported by different workers reflect both differences between cell types as well as differences between experimental protocols. Different concentrations of Mtx may result in different levels of inhibition of enzymes and/or different changes in nucleotide pools. This has been shown to occur when 5-Fu toxicity in two different cell lines was analysed [20].

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