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Effect of methotrexate and 5-fluorouracil on de novo thymidylate synthesis in human colon carcinoma cell line, Caco-2

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Although colon cancers respond poorly to chemotherapeutic agents, 5-fluorouracil (5-FU) is considered the most effective single agent in the treatment of advanced colon cancer, whereas methotrexate has been reported as an ineffective agent. But both 5-FU and methotrexate inhibit de novo thymidylate synthesis. In the present study we assessed the sensitivity of folate-dependent thymidylate synthesis to methotrexate and 5-FU in Caco-2, a human colon carcinoma cell line. Sensitivity was assessed indirectly, by the deoxyuridine suppression test and directly, by the degree of inhibition of [^{3}H]deoxyuridine incorporation into deoxyribonucleic acid (DNA). Methotrexate or 5-fluorodeoxyuridine resulted in a significant decrease in suppression of [^{3}H]thymidine incorporation by exogenous deoxyuridine. [^{3}H]deoxyuridine incorporation was also inhibited by the two agents. Inhibition was dose dependent and 50% inhibition occurred at about 2.5 μmol/L methotrexate and 25 μmol/L 5-FU. In a second study, the effect of methotrexate and 5-FU on [^{3}H]deoxyuridine incorporation into DNA was assessed under conditions in which a Caco-2 cell monolayer was exposed to the agents either at the apical or at the basolateral membrane side. Under these conditions, inhibition was also dose dependent and cells were more sensitive to basolateral exposure to both methotrexate and 5-FU ($P < 0.05$). The data suggest that both methotrexate and 5-FU are effective inhibitors of thymidylate synthesis in Caco-2 cells. Determining the degree of inhibition of deoxyuridine incorporation into DNA is an effective method for evaluating these agents' effect on de novo thymidylate synthesis. Further studies are required to determine if these inhibitory effects also hold for colon cancer tissue biopsies and whether the reported differences in therapeutic efficacy between the two agents can be manifested in vitro. (J. Nutr. Biochem. 7:513–517, 1996.)

Keywords: colon cancer; thymidylate synthesis; deoxyuridine suppression test; [^{3}H]deoxyuridine incorporation into DNA; Caco-2; methotrexate; 5-FU

Introduction

Colon cancer is a major cause of cancer-associated morbidity and mortality.[1] Patients with advanced unresectable colon cancer still have a poor prognosis due to few advances in chemotherapy.[2] Since its clinical introduction in 1957, 5-fluorouracil (5-FU) has been the mainstay of chemotherapy for colon cancer. Although the response rate to 5-FU as a single agent is 10 to 25%, recent studies have shown that the addition of modulating agents, such as folinic acid, α-2a-interferon or levamisol to 5-FU improves the response rate of colon cancer chemotherapy.[3–5] In contrast, methotrexate (MTX) has been regarded as an ineffective single chemotherapeutic agent for colon cancer with 5 to 15% response rates.[6,7] Even when used in combination with 5-FU, MTX's effectiveness is still in doubt.[8,9]

It is unclear why MTX is less effective than 5-FU in the

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treatment of colon cancer. Both MTX, an inhibitor of dihydrofolate reductase, and 5-FU, an inhibitor of thymidylate synthase, block de novo folate-dependent thymidylate synthesis.[10,11] Although resistance to MTX in several types of leukemia, breast cancer, and soft tissue sarcomas[12,13,14] has been well documented, the response of colon cancer cells to MTX has not been clearly demonstrated.

The present study was undertaken to assess the effect of MTX and 5-FU on the de novo synthesis of thymidylate in the human colon carcinoma cell line, Caco-2. De novo thymidylate synthesis was assessed by the deoxyuridine suppression test[15,16] and the extent of [^{3}H]deoxyuridine ([^{3}H]dU) incorporation into DNA. The deoxyuridine suppression test is a functional test of folate status as it assesses the degree of suppression of [^{3}H]thymidine incorporation into DNA by exogenous dU. The degree of inhibition of [^{3}H]dU incorporation into DNA directly reflects the inhibition of de novo thymidylate synthesis.

Methods and materials

Cell culture

The cell line Caco-2, which was established from a moderately well differentiated colon adenocarcinoma, is the only known colon cancer cell line to spontaneously differentiate, and can be grown to form monolayers with tight junctions.[17] Caco-2 cells (ATCC, Rockville, MD USA) were maintained at 37°C in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) containing 1% L-glutamine (BioWhittaker, Walkersville, Maryland USA), 1% non-essential amino acids, 1% sodium pyruvate, 1×10^5 unit/L penicillin, 100 mg/L streptomycin, and 50 mg/L gentamicin (Gibco, Grand Island, NY USA), and 10% fetal bovine serum (HyClone, Logan, Utah USA).[18] Cells were grown in 75 cm^2 T-flasks (Falcon, Oxnard, CA USA) in a 5% CO_2-95% air atmosphere at 90% relative humidity, were passaged every 4 days, and were used between passage 80 and 110.

Effect of MTX and 5-FdU on thymidylate synthesis determined by the deoxyuridine suppression test

Caco-2 cells were harvested on the 6th day after passage using 0.05% trypsin and 0.53 mmol/L EDTA. Cells ($0.6–0.8 \times 10^6$) in Hank's balanced salt solution (pH 7.4, 0 μmol/L folic acid) containing 1% glutamine were incubated for 30 min at 37°C with 12.5 μmol/L MTX or 6.25 μmol/L 5-FdU (Sigma, St. Louis, MO USA). Then 300 μmol/L dU was added and the incubation was continued for 30 min before the addition of 0.6 μmol/L [^{3}H]thymidine (Amersham, Arlington Heights, IL USA) after which the incubation was continued for an additional 60 min. The reaction was stopped by adding 3 mL ice-cold saline and the cells were washed three times with ice-cold saline. DNA was precipitated with 2 mL 10% trichloroacetic acid (Sigma, St. Louis, MO USA), 0.5 mL Soluene 350 (Packard, Downers Grove, IL USA) was added to the DNA and the mixture was transferred into a scintillation vial with 10 mL of scintillation cocktail (Fisher, Pittsburgh, PA USA). [^{3}H]thymidine in DNA was counted in a liquid scintillation counter. Deoxyuridine suppression was expressed as the percentage of labeled [^{3}H]thymidine incorporation into DNA in the presence of dU divided by incorporation in the absence of dU.[15] All incubations were done in triplicate.

Effect of MTX and 5-FU on [^{3}H]dU incorporation into DNA

Caco-2 cells were harvested on the 4th day after passage and 2×10^5 cells were seeded in 2 cm^2 wells of a Multiwell tissue culture plate (Becton Dickinson, Lincoln Park, NJ USA) with 2 mL DMEM containing 20% fetal bovine serum (FBS). At day 4, cells were washed three times with 0.9% saline and incubated for 30 min at 37°C with MTX or 5-FU at various concentrations in 2 mL DMEM (pH 7.4, 9 μmol/L folic acid) containing 10% FBS. Then 0.05 μmol/L [^{3}H]dU (37 kBq, Amersham, Arlington Heights, IL USA) was added and the incubation was continued for 90 min at 37°C in a shaking water bath. After removing the incubation media the wells were rinsed three times with 0.9% ice-cold saline. Thereafter, the cells were scraped and suspended in 2 mL 10% trichloroacetic acid. Precipitated DNA was collected by centrifugation for 10 min at 900g. The DNA was dissolved in 0.5 mL Soluene 350, transferred to a scintillation vial using 10 mL of scintillation cocktail, and counted in a liquid scintillation counter.[15] All incubations were done in triplicate.

Apical and basolateral accessibility of MTX and 5-FU

Caco-2 cells were seeded in 4.7 cm^2 Transwell cell culture chamber inserts (tissue culture treated, 0.4 μm pore polycarbonate membrane type, Costar, Cambridge, MA USA) at a density of 43,000 cells/cm^2 with 2 mL DMEM containing 20% FBS in the upper and lower compartments.[19] Confluent monolayers were formed 6 to 7 days after seeding and the monolayers became intact after 11 to 14 days.[17,18] Studies were performed at 15 days of culture. Intactness of monolayer was confirmed by phenol red test.[20] Both sides of monolayers were washed three times with 0.9% saline. The monolayer was preincubated in DMEM (pH 7.4, 9 μmol/L folic acid) containing 10% FBS for 30 min with various concentrations of MTX or 5-FU added either to the lower compartment (basolateral side) or the upper compartment (apical side).[17] Thereafter, a solution containing [^{3}H]dU (37 kBq) was added to the upper compartment and the cells were incubated for 90 min at 37°C. After incubation, both sides of the membrane were washed three times with 0.9% cold saline. The membrane was detached from the inserts, crushed into small pieces, and transferred into a culture tube. Two mL of 10% trichloroacetic acid solution was added and the suspension was centrifuged for 10 min at 900g. After removal of the supernatant, 0.5 mL of Soluene 350 was added to solubilize the DNA and membrane. After solubilization, the labeled DNA was counted in a scintillation counter. All incubations were done in triplicate.

Statistical analysis

Statistical differences between the mean values of triplicate measurements were determined by Student's t test with $P < 0.05$ considered to be significant (Systat, Systat Inc, Evanston, IL USA).

Results

Figure 1 shows that preincubation of Caco-2 cells with 12.5 μmol/L MTX and preincubation with 6.25 μmol/L 5-FdU significantly decreased the suppression of [^{3}H]thymidine incorporation into DNA at 300 μmol/L dU ($P < 0.05$). *Figure 2* shows that both MTX and 5-FU are inhibitors of [^{3}H]dU incorporation into DNA of Caco-2 cells. The inhibition is dose-dependent with 50% inhibition occurring at about 2.5 μmol/L MTX and 25 μmol/L 5-FU.

The sensitivity of cells to apical or basolateral exposure to drug was also evaluated by determining the degree of inhibition of [^{3}H]dU incorporation into DNA. *Figure 3* shows that both sides are accessible to either agent, although

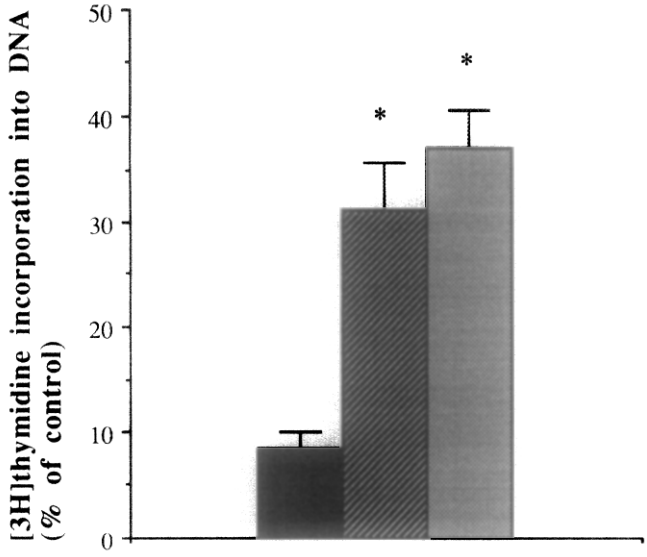

Figure 1 Effect of MTX and 5-FdU on the deoxyuridine suppression test in Caco-2 cells. Preincubation with MTX and dU (hatched bar) and preincubation with 5-FdU and dU (dotted bar) were significantly decreased the suppression of [^{3}H]thymidine incorporation into DNA compared with preincubation with dU alone (solid bar) (*P < 0.05). The concentrations of MTX, 5-FdU, and dU were 12.5, 6.25, and 300 μmol/L, respectively.

not to the same extent. For MTX, 50% inhibition required about 1 μmol/L after basolateral exposure and about 50 μmol/L after apical exposure. For 5-FU, 50% inhibition required about 500 μmol/L after basolateral exposure and twice that amount after apical exposure.

The data in *Figure 3* also show that basolateral exposure to either agent was associated with a greater degree of inhibition of [^{3}H]dU incorporation into DNA than apical exposure.

Discussion

5-FU is regarded as the most effective agent for systemic treatment in patients with colon cancer. On the other hand, MTX has been regarded as an ineffective agent in colon cancer chemotherapy since Hall reported a higher response to 5-FU in 1962.[21] But 5-FU and MTX have the same major site of action, de novo folate-dependent thymidylate synthesis. MTX inhibits thymidylate synthesis indirectly by blocking dihydrofolate reductase whereas 5-FU, after conversion to 5-fluorodeoxyuridylate, inhibits thymidylate synthase.[22] Many possible mechanisms of MTX resistance have been reported in other cancers including decreased MTX transport into the cell and/or increased efflux from the cell, decreased intracellular polyglutamation of MTX, alteration of dihydrofolate reductase.[23,24] To explore the resistance mechanism in colon cancer, we first investigated the effect of MTX and 5-FU on a colon cancer cell by determining the effect of these agents on de novo thymidylate synthesis.

In 1991, Cravo et al. reported that the deoxyuridine suppression test was useful for detecting folate deficiency in the colonic epithelium.[15] However, MTX did not change the degree of suppression of [^{3}H]thymidine incorporation into DNA by dU in rat colonocytes even at a 1 mmol/L concentration, whereas 5-FU (50 μmol/L) decreased the degree of suppression by added dU.[15] They suggested impaired transport of MTX across the cell membrane as a possible mechanism for the lack of MTX effect. In our study, we investigated the effect of MTX and 5-FU on thymidylate synthesis in a colon cancer cell line by the deoxyuridine suppression test and the degree of inhibition of [^{3}H]dU incorporation into DNA.

Our data show that both agents were inhibitors of thymidylate synthesis in the Caco-2 cell. Using cell monolayers on a solid phase, in which cells were exposed only via their apical membrane, inhibition was dose-dependent and highly effective with 50% inhibition around 2.5 μmol/L MTX and 25 μmol/L 5-FU. Under conditions of monolayer culture on a transwell membrane, in which cells were ex-

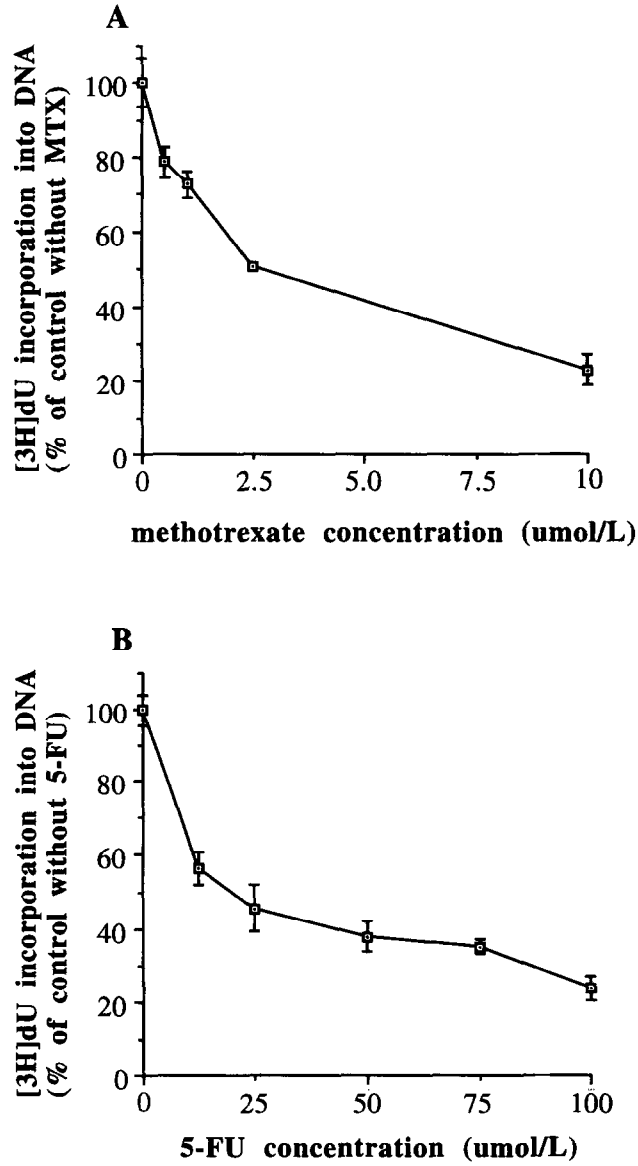

Figure 2 Effect of MTX and 5-FU on de novo thymidylate synthesis in Caco-2 cells determined by [^{3}H]dU incorporation into DNA. (A) [^{3}H]dU incorporation at 0, 0.5, 1, 2.5, and 10 μmol/L MTX, (B) [^{3}H]dU incorporation at 0, 10, 25, 50, 75, and 100 μmol/L 5-FU. Each value represents the mean ± SEM of triplicates.

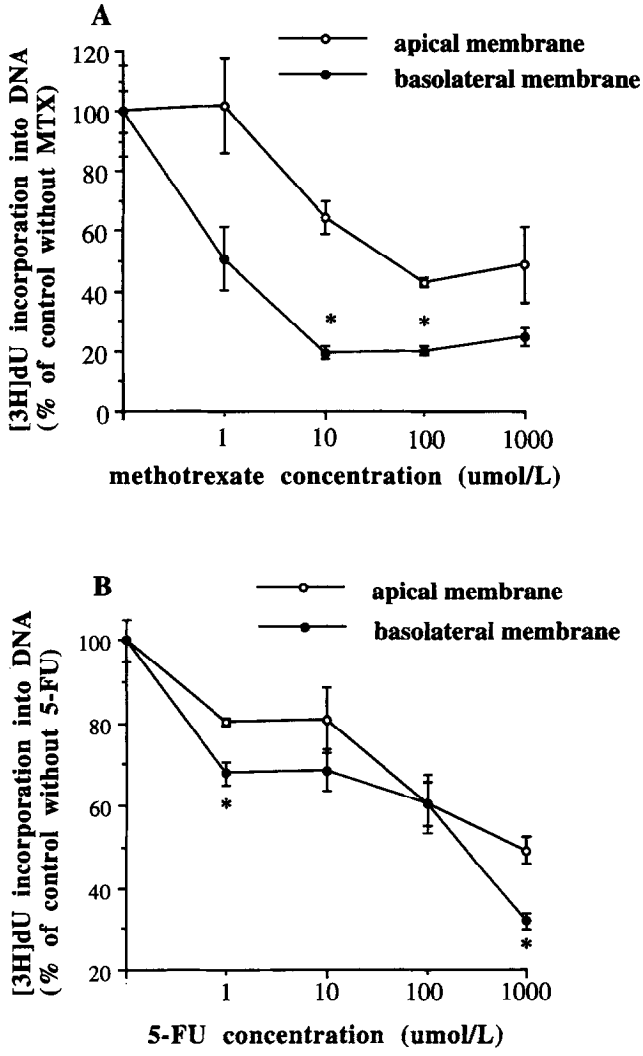

Figure 3 Accessibility of MTX and 5-FU through apical and basolateral membrane in Caco-2 cell. (A) [^{3}H]dU incorporation into DNA at 0, 1, 10, 100, and 1000 μmol/L MTX, (B) [^{3}H]dU incorporation into DNA at 0, 1, 10, 100, and 1000 μmol/L 5-FU. Each value represents the mean ± SEM of triplicates (*$P < 0.05$).

posed to both influx and efflux via either apical or basolateral membranes, inhibition via apical transport was less effective and differed depending on the agent. Under these conditions, MTX exposure via the apical membrane was ineffective, even at high concentrations, but MTX exposure via the basolateral membrane was highly effective with 50% inhibition around 1 μmol/L. This suggests that defective transport into the cell across the basolateral membrane is not the cause of MTX resistance in colon cancer. In fact, there are differences in transport mechanism between apical and basolateral membranes of intestine, particularly for MTX: basolateral transport likely occurs via the reduced folate carrier, whereas apical transport is via two different mechanisms, passive diffusion and facilitated diffusion via a low-affinity carrier.[25,26,27]

Transmembrane transport of 5-FU is difficult to characterize kinetically due to the rapidity of drug permeation and its intracellular conversion to nucleotides.[28,29] In this study 5-FU transport via the basolateral membrane was more effective than via the apical membrane but the difference was not as large as that observed with MTX.

In conclusion, our data indicate that Caco-2, a cell line derived from a human colon carcinoma, is sensitive to MTX and 5-FU. Determining the degree of inhibition of [^{3}H]dU incorporation into DNA is an effective method for evaluating these agents' effects on de novo thymidylate synthesis. This should allow us to extend our investigations to biopsies or surgical specimens from colon cancer patients.

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