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Methotrexate Induced Differentiation in Colon Cancer Cells is Primarily Due to Purine Deprivation

R. Singh,[1] A.A. Fouladi-Nashta,[1] D. Li,[1] N. Halliday,[2] D.A. Barrett,[2] and K.D. Sinclair[1]*

[1]School of Biosciences, University of Nottingham, Sutton Bonington, LE12 5RD, United Kingdom
[2]School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, United Kingdom

Abstract The folate antagonist methotrexate (MTX) inhibits synthesis of tetrahydrofolate (THF), pyrimidines and purines, and induces differentiation in several cell types. At 1 μM, MTX reduced proliferation and induced differentiation in HT29 colon cancer cells; the latter effect was augmented ($P < 0.001$) by thymidine (100 μM) but was reversed ($P < 0.001$) by the purines, hypoxanthine (Hx; 100 μM) and adenosine (100 μM). In contrast 5-fluoro-uracil (5-FU), a specific thymidylate synthase (TS) inhibitor, had no effect on differentiation, suggesting that MTX-induced differentiation is not due to a reduction in thymidine but to the inhibition of purine biosynthesis. Inhibition of cyclic AMP (cAMP) by RpcAMP (25 μM) further enhanced ($P < 0.001$) MTX induced differentiation, whereas the cAMP activator forskolin (10 μM) reversed ($P < 0.001$) MTX induced differentiation. These observations implicate a central role of adenosine and cAMP in MTX induced differentiation. By combining Western blot analysis with liquid chromatography-mass spectrometry (LC-MS), methionine synthase (MS), and HPLC analyses we also reveal both the expression and activity of key enzymes (i.e. MS, s-adenosylhomocysteinase, cystathionine β-synthase and ornithine decarboxylase) regulating methyl cycle, transsulfuration and polyamine pathways in HT29 colon cancer cells. At 1 μM, MTX induced differentiation was associated with a marked reduction in the intracellular concentrations of adenosine and, consequently, S-adenosylmethionine (SAM), S-adenosylhomocysteine, polyamines and glutathione (GSH). Importantly, the marked reduction in methionine that accompanied MS inhibition following MTX treatment was non-limiting with respect to SAM synthesis. Collectively, these findings indicate that the effects of MTX on cellular differentiation and single carbon metabolism are primarily due to the intracellular depletion of purines. J. Cell. Biochem. 99: 146–155, 2006.

Key words: differentiation; colon cancer; purines; methotrexate

The epithelial cells of the mammalian intestinal tract are hierarchically arranged so that cells become more differentiated as they ascend the crypt-villus axis. The process is maintained and regulated by a population of intestinal stem cells, which reside within the crypts of the small and large intestine and generate all the intestinal epithelial lineages [Vidrich et al., 2003]. Intestinal differentiation is accompanied by changes in morphology, temporal and spatial-specific expression of genes, such as the brush border enzymes sucrase-isomaltase and intestinal alkaline phosphatase (ALP), and is regulated by a myriad of biochemical pathways [Leedham et al., 2005]. Aberrations in the biochemical signaling cascades within stem cells play an integral part in the development of gastrointestinal malignancy.

Several compounds such as methotrexate (MTX), butyrate, retinoids, and Vitamin D can induce differentiation in colon and other cancer cells [Spira and Carducci, 2003]. There are probably mechanistic differences in how the various agents induce differentiation in cancer cells. However, the overall process involves the reversion of malignant tumor cells into more benign forms, in which cellular proliferation is lowered resulting in reduced tumor growth. MTX has been used to treat millions of patients with malignant and autoimmune diseases such as rheumatoid arthritis and has been in clinical use for over 50 years [Fairbanks et al., 1999]. MTX enters the cell via a reduced folate carrier

*Correspondence to: Dr. K.D. Sinclair, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK.
E-mail: kevin.sinclair@nottingham.ac.uk
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and is converted into a polyglutamated form by folypolyglutamyl synthetase [Bosson, 2003]. The polyglutamated form is retained in the cell and inhibits not only dihydrofolate reductase but has an increased affinity for certain folate-dependent enzymes such as thymidylate synthase (TS) and enzymes involved in purine synthesis [Kimura et al., 2004]. MTX targets enzymes involved in the metabolism of three specific tetrahydrofolates (THFs) that play essential roles as single carbon carriers involved in the synthesis of DNA precursors [McGuire, 2003]. The first THF, 10-formyltetrahydrofolate, donates its single carbon group for the synthesis of purines in reactions mediated by glycineamide ribonucleotide (GAR) transformylase and aminoimidazolecarboximide ribonucleotide (AICAR) transformylase. The second THF, 5, 10-methylenetetrahydrofolate, donates its single carbon group for the reductive methylation reaction mediating conversion of dUMP to thymidylate. The third THF, 5-methyltetrahydrofolate, donates methyl groups in the methylation of homocysteine to methionine. Inhibition of GAR and AICAR transformylases, TS and dihydrofolate reductase is central to the pharmacological actions of MTX. MTX, therefore, inhibits multiple enzyme targets, perturbs methyl and folate cycles and inhibits purine and pyrimidine synthesis.

The present study sought to demonstrate which of these effects of MTX are involved in differentiation of HT29 colon cancer cells and also to identify the key mechanisms and signaling pathways involved in MTX-induced differentiation. We also report, for the first time in HT29 colorectal cells, the presence of active methyl cycle, transsulfuration and polyamine pathways by demonstrating the expression of key enzymes, and by monitoring the relative changes in methyl cycle metabolites following MTX treatment using a combination of liquid chromatography-mass spectrometry (LC-MS) and HPLC analyses.

MATERIALS AND METHODS

Cell Culture

We used the well characterized human colon cancer cell line, HT29, as a model for intestinal differentiation. This cell line displays a multipotent phenotype, characterized by differentiation into a polarized monolayer with the expression of the brush-border enzyme ALP after treatment with the short chain fatty acid, sodium butyrate or MTX [Heerdt et al., 1994]. The cell line has been extremely useful in delineating potential pathways resulting in an enterocyte-like phenotype and therefore has been extensively used as a model for intestinal cell differentiation [Hodin et al., 1996; Matthews et al., 1998; Cohen et al., 1999; Taupin and Podolsky, 1999]. These cells were obtained from the European Collection of Animal Cell Cultures at the Public Health Laboratory Service (Porton Down, Wiltshire, UK). The cells were grown in monolayers and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were initially cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 ng/ml insulin. The cells were grown in 75 cm^2 flasks. The medium was replaced after 24 h with fresh DMEM containing 5% FCS and appropriate concentrations of test reagents as indicated in the figure legends and the cells were harvested after 48 h. Thymidine, hypoxanthine (Hx), adenosine, THF, MTX, 9-fluorenylmethyl chloroformate, glycine, forskolin, PD98059, wortmannin and staurosporine used in this study were all purchased from Sigma (Sigma, Poole, Dorset, UK).

Cell Proliferation and Differentiation

The cells were lysed in 250 μl 0.25% NP-40 in phosphate buffer saline (PBS). The lysates were stored at −20°C until determination of ALP activity and protein concentration. The activity of the ALP enzyme was quantified using Sigma kit 204 (Sigma Ltd, Poole, Dorset, UK) in a 96-well assay plate with a microplate reader (Bio-Rad, Hemel Hemstead, UK). The method is based on the ability of the enzyme to convert *p*-nitrophenyl phosphate to *p*-nitrophenol, which in alkaline solution presents as a yellow color and can be read at 410 nm. For ALP staining, the cells were cultured on 13 mm sterile glass cover slips (Fisher Scientific, Loughborough, UK) in 24 well culture dishes (Nunc, Hereford, UK) at 1.5×10^5 cells/ml in a humidified atmosphere of 5% CO_2 in air at 37°C. The media was replaced with fresh media (DMEM with 5% FCS) containing appropriate concentrations of test reagents, as indicated in the figure legends,

and the cells were harvested after 48 h. Assessments of differentiation were determined in triplicate. The culture medium was removed from the cover slips after 48 h and the cover slips quickly washed with cold PBS, fixed for 5 min in cold acetone (4°C) and stored at −20°C until required for ALP staining. The cells were stained for ALP activity using SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma Ltd, Poole, Dorset, UK). One tablet was dissolved in 10 ml of distilled water by stirring and 250 μl of the solution per coverslip was added. Samples were incubated at room temperature for an hour and the reaction was stopped following the addition of PBS. Samples were washed twice with cold PBS, and mounted in DPX (BDH, Poole, Dorset, UK). The stained cells were photographed using an imaging microscope (Olympus BH2, London, UK). To assess cell proliferation the harvested cells were resuspended in 1 ml of DMEM and to a 200 μl aliquot were added 500 μl DMEM and 300 μl trypan blue. The cells were mixed gently and counted using a cytometer.

Western Blot Analysis

Cell lysates were mixed with SDS–PAGE sample buffer (62.5 mM Tris [pH 6.8] containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, and 0.001% (w/v) bromophenol blue) and boiled for 5 min. The polypeptides were then separated on an 8% SDS-polyacrylamide gel. The separated polypeptides were electrotransferred to nitrocellulose membranes for 1 h at 100 V in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. After transfer, the membrane was blocked with PBS containing 5% (w/v) dry milk and 0.1% (v/v) Tween-20 for 1 h, and then incubated with primary antibody for 1 h. The membrane was then washed five times with PBS containing 0.1% Tween-20, followed by incubation with peroxidase-conjugated secondary antibody for 1 h. The membrane was washed again and the bound antibody was detected using an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, Bucks, UK). The blots were stripped and reprobed with β-actin to confirm equal loading. Densitometry was performed using Quantity One software (Biorad, Hemel Hemstead, UK). Total protein was measured using the method of Bradford with bovine serum albumin as the standard protein [Bradford, 1976].

HPLC Analysis of Thiols and Polyamines

Cell pellets were suspended in 700 μl phosphate buffered saline containing a mixture of protease inhibitor cocktail (Sigma Cat. No. P2714) and the suspensions sonicated on ice using three 20-s pulses to lyse the cells, followed by centrifugation at 18,000*g* for 15 min. The supernantant fraction was used to measure intracellular thiols using Agilent 1100 HPLC (Agilent Technologies, Stockport, Cheshire, UK) using methods described previously [Pfeiffer et al., 1999]. Briefly 100 μl of cell lysate was mixed with reducing agent containing tris (2-carboxyethyl) phosphine, phosphate buffered saline and cystamine as an internal standard, and incubated for 30 min at room temperature, after which 180 μl of 10% (w/v) trichoroacetic acid containing 1 mmol/L EDTA was added for deproteinization. After the sample was centrifuged for 10 min at 13,000*g*, 100 μl of supernatant was added to an autosampler vial containing 20 μl of 1.55 mol/L NaOH; 250 μl of 0.125 mol/L borate buffer containing 4 mmol/L EDTA, pH 9.5; and 100 μl of 1 g/L SBD-F in borate buffer. The sample was then incubated for 60 min at 60°C. HPLC was carried out on an Agilent 1100 series system (Agilent Technologies, Stockport, Cheshire, UK) equipped with a fluorescence detector (385 nm excitation, 515 nm emission). Separation of SBD-F deravitized thiols was performed on a Prodigy ODS2 analytical column, 150 × 3.2 mm, 5 μm (Phenomenex, Macclesfield, Cheshire, UK) with an Adsorbosphere C_{18}, 3-cm guard column, using a 25 μl injection volume and 0.1 mol/L sodium acetate buffer pH 5.38 containing 3.05% methanol as mobile phase at a flow rate of 0.7 ml/min and a column temperature of 29°C.

Polyamine analysis was performed as described previously [Ekegren et al., 2004]. Briefly, to a homogenate containing 100 μg protein, 60 μl borate buffer (0.2 M, pH 9.0) and 5 μl of internal standard (1.6-DAH; 30 nmol/ml) were added. The derivitization was performed with 200 μl 9-fluorenylmethyl choroformate (0.01 M in acetone). After 90 s the excess FMOC was allowed to react with 240 μl freshly prepared glycine reagent [0.04 M glycine dissolved in 0.2 M borate buffer (pH 9.0): Acetone, 50/50 v/v] and mixed for 45 s followed by addition of dilution buffer [0.05 M sodium acetate buffer (pH 4.2): Acetonitrile 30/70, v/v]. After 45 s the total volume was adjusted to 800 μl with 200 μl

of water. The separation was carried out on a C_{18} reverse phase column, protected by a 3-cm guard column at a flow rate of 1.2 ml/min. The separation was achieved using a gradient comprising two buffer systems. Buffer A consisted of 0.05 M sodium acetate buffer (pH 4.2): Acetonitrile 80/20, v/v. Buffer B consisted of 0.05 M sodium acetate buffer (pH 4.2): Acetonitrile 5/95, v/v. Both polyamines and thiols were quantified using chromatographic software (Chemstation Plus, Agilent Technologies, Stockport, Cheshire, UK).

LC-MS Analysis of Metabolites

S-adenosyl methionine, s-adenosyl homocysteine, adenosine, and methionine were analyzed by quantitative liquid chromatography tandem electrospray ionization mass spectrometry (LC-MS-MS). The metabolites were derivatised as N (O, S)-*i*-butylcarbonyl *i*-butyl esters using *i*-butyl chloroformate. The method enabled fast and simultaneous esterification of the carboxylic and other protic function groups such as hydroxyl, thiol and amine groups. Briefly, 90 μl water containing two internal standards was added to the freeze-dried sample of extracted metabolites. This was followed by the addition of 40 μl iso-butanol/pyridine mixture (3:1 v/v), 10 μl iso-butylchloroformate and 200 μl dichloromethane/tert-butylmethyl-ether (1:2 v/v) with mixing at each stage. One hundred fifty microliters of upper organic phase was removed, and evaporated to dryness under a stream of nitrogen gas. The derivatised metabolites were then redissolved in 100 μl LC mobile phase (80% v/v methanol in 10 mM ammonium formate) prior to LC-MS–MS analysis. LC separation was done on an Agilent 1100 system (Agilent, Waldbron, Germany) using a mobile phase (80% v/v methanol in 10 mM ammonium formate) at a flow rate of 200 μl/min using a LUNA ODS C18(2) column (150×2.0 mm, 5 μm particle size) (Phenomenex, Macclesfield, UK), maintained at 30°C. The MS system was a Waters Micromass Quattro Ultima triple quadrupole (Waters, Manchester, UK), used in positive ion multiple reaction monitoring scan mode to monitor specific precursor/product ions for each metabolite. The ESI source was operated in the positive-ion mode at a capillary voltage of 2.81 V, and a cone voltage of 35.0 V. Nitrogen was used as the nebulizing gas at a flow rate of 155 L/h, and as the desolvation gas at a flow rate of 765 L/h. The ion source was at a temperature of 125°C, and the desolvation temperature was maintained at 350°C. The concentrations of individual metabolites were calculated from calibration lines using the peak area ratio to internal standard.

Data Analysis

Each experiment was conducted in triplicate. The data were analyzed by one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons. All results are expressed as mean ± SEM, and a P-value of <0.05 was considered statistically significant.

RESULTS

Thymidine Augments Whereas Hypoxanthine, Adenosine and Tetrahydrofolate Inhibit Methotrexate Mediated Induction of ALP Activity

At 1 μM, MTX treatment resulted in enterocyte-like differentiation by 48 h as confirmed by induction of the brush-border enzyme ALP (Fig. 1A). MTX treated cells were enlarged and rounded with intense ALP staining relative to controls. To determine whether pyrimidine or purine nucleotides affected MTX-induced intestinal cell differentiation we first co-administered MTX with thymidine for 48 h. The cells were harvested after 48 h and lysed and the extracts assayed for ALP activity. Thymidine (100 μM) co-administration resulted in 1.4-fold augmentation ($P < 0.001$) of MTX mediated induction of ALP activity (Fig. 1A and B). Five-fluoro uracil (5-FU; 2 μM), a specific inhibitor of TS, failed to enhance ALP activity on its own or MTX-induced ALP activity (Fig. 1C). In contrast, supplementation with the purines Hx (100 μM) (Fig. 1A and B) or adenosine (100 μM) (Fig. 1C) almost completely abolished ($P < 0.001$) MTX-mediated induction of ALP activity. These results suggest that MTX-induced differentiation is mainly due to inhibition of purine synthesis. Interestingly, THF supplementation (50 μM) also inhibited MTX-mediated ALP induction (Fig. 1B).

Inhibition of Cyclic AMP, MEK-ERK, and PI-3 Kinase Enhances Methotrexate Mediated ALP Activity

Since adenosine is required for cyclic AMP (cAMP) synthesis, and our data show that the

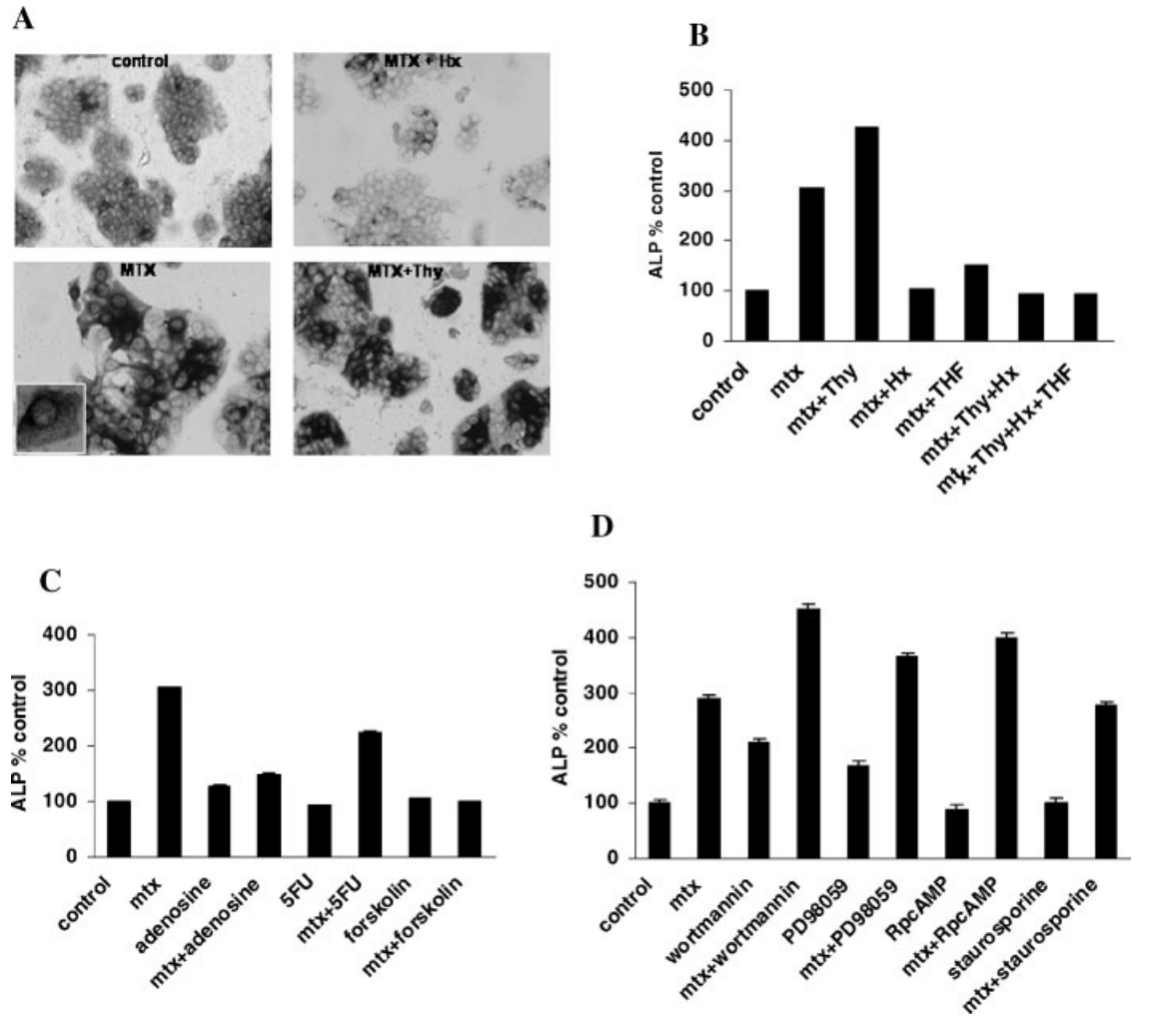

Fig. 1. **A**: Alkaline phosphatase (ALP) staining (10 × magnification) of untreated (control) HT29 cells or HT29 cells treated with methotrexate (MTX; 1 μM) alone or in combination with hypoxanthine (Hx; 100 μM) or thymidine (Thy; 100 μM). Hx inhibited MTX-induced ALP staining whereas thymidine augmented it. Inset shows a fully differentiated cell exhibiting intense ALP staining. ALP induction (relative to control) in HT29 cells following (**B**) treatment with MTX alone or MTX with various combinations of Thy, Hx, and tetrahydrofolate (THF; 50 μM); (**C**) treatment with MTX, adenosine (100 μM), 5-fluro-uracil (5FU; 2 μM) and forskolin (10 μM) alone or in combination with MTX; (**D**) treatment with MTX, wortmannin (1 μM), PD98059 (2 μM), RpcAMP (25 μM) or staurosporine (10 nM), alone or in combination with MTX.

depletion of adenosine leads to MTX-induced intestinal cell differentiation, we next determined if cAMP was involved in this process. HT29 cells were treated with 1 μM MTX for 48 h in combination with cAMP activator, forskolin (10 μM). Forskolin reversed ($P < 0.001$) MTX-mediated induction of ALP activity but had no effect on ALP activity on its own (Fig. 1C). In contrast, RpcAMP (25 μM), a specific inhibitor of cAMP, augmented by 1.3 fold ($P < 0.001$) MTX-induced ALP activity (Fig. 1D). Next, HT29 cells were treated for 48 h with inhibitors of PKC (staurosporine; 10 nM), PI3-kinase (P13-K) (wortmannin; 1 μM) or the MAP kinase pathway (MEK-ERK; PD98059; 2 μM), alone or in combination with 1 μM MTX. Both wortmannin and PD98059 treatments resulted in 2.0- and 1.7-fold increase ($P < 0.001$) respectively in ALP activity after 48 h of treatment compared to DMSO control (Fig. 1D). Furthermore, these inhibitors, in combination with MTX, increased ($P < 0.001$) ALP activity by 1.5 and 1.2 fold, respectively, compared with MTX alone. In contrast, the PKC inhibitor, staurosporine, had no effect on ALP activity alone or in combination with MTX (Fig. 1D).

Methotrexate and Proliferation of HT29 Cells

As the antifolate, MTX, is known to inhibit the proliferation of various cell types, so we next

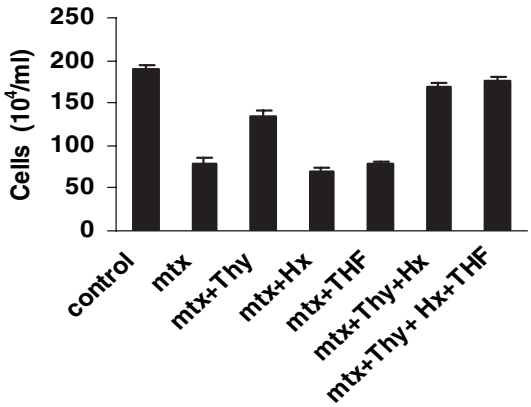

Fig. 2. At 1 μM, MTX-induced inhibition of proliferation is partially reversed by thymidine (Thy; 100 μM) and is fully restored by a combination of thymidine and Hx (100 μM), whereas Hx or THF (50 μM) proved ineffective.

determined if exogenous THF could reverse the anti-proliferative effects of MTX in our colon cancer cells. The results show that, at 1 μM, MTX-induced inhibition of proliferation was fully reversed by the combination of thymidine (100 μM) and Hx (100 μM), and was partially restored ($P < 0.001$) by thymidine alone (Fig. 2). However, neither Hx nor THF (50 μM) when added alone were able to reverse this MTX-induced inhibition of cell proliferation.

Methotrexate Reduces Intracellular Adenosine, Methionine, SAM, and SAH

Since supplementation with purines, Hx or adenosine reversed MTX mediated induction of ALP activity we next determined, by LC-MS, the effect of MTX (1 μM) on intracellular concentrations of adenosine in HT29 colon cancer cells. The results show that MTX caused a dramatic decline (by 89%) ($P < 0.005$) in intracellular adenosine (Fig. 3A). Hx (100 μM) alone partially reversed this MTX induced decline ($P < 0.05$) in adenosine whereas thymidine (100 μM) or THF (50 μM) had no effect. However, the combination of thymidine and Hx completely reversed ($P < 0.001$) the MTX-induced decline in intracellular adenosine concentrations. We next determined the effect of MTX on intracellular methionine concentrations. MTX treatment resulted in a sharp decrease ($P < 0.001$) in intracellular methionine (Fig. 3B), an effect, which was only partially reversed ($P < 0.001$) by a combination of thymidine and Hx. Co-administration of MTX with Hx or thymidine, however, failed to restore basal concentrations of intracellular methionine. Since both methionine and adenosine are required for the synthesis of universal methyl donor SAM, and its demethylated product and negative regulator, SAH, we next determined the relative changes in SAM and SAH in response to MTX treatment. MTX treatment resulted in a 37 and 57% decrease in intracellular concentrations of SAM ($P = 0.002$) and SAH ($P < 0.01$) respectively, as determined by LC-MS (Fig. 3C). Interestingly, compared to MTX alone, co-administration of MTX with thymidine failed to increase intracellular SAM and SAH concentrations. However, supplementation with either Hx or a combination of thymidine and Hx significantly increased intracellular SAM and SAH concentrations compared to MTX alone. Furthermore, when MTX was co-administered with a combination of thymidine, Hx and THF, a complete recovery of intracellular SAM and SAH concentrations was observed ($P < 0.01$). MTX supplementation with THF, however, failed to enhance intracellular SAM and SAH concentrations.

Methotrexate Reduces Intracellular Glutathione and Polyamines in HT29 Cells

S-adenosylmethionine is an aminopropyl donor for polyamine synthesis [Moinard et al., 2005] and a positive regulator of cystathionine β-synthase [Stipanuk, 2004]. This enzyme is involved in the conversion of homocysteine into cystathionine, which is eventually converted into cysteine and glutathione (GSH). Consequently, we sought to determine the effects of MTX on intracellular GSH and polyamine (spermidine and spermine) concentrations. Analysis by HPLC revealed that, relative to control, MTX treatment (1 μM) resulted in a sharp decrease ($P < 0.001$) in intracellular GSH (by 72%), spermidine (by 48%) and spermine (by 55%) concentrations (Figs. 4A and B). This reduction in intracellular GSH and polyamine concentrations was reversed following supplementation with Hx (100 μM) alone; but neither thymidine (100 μM) nor THF (50 μM) proved effective in this regard. MTX-mediated decreases in intracellular SAM concentrations, therefore, appear to downregulate GSH and polyamine synthesis.

Methotrexate Affects Methyl Cycle and Polyamine Pathway Enzyme Expression

In an attempt to attribute the aforementioned metabolic effects of MTX to either its anti-folate

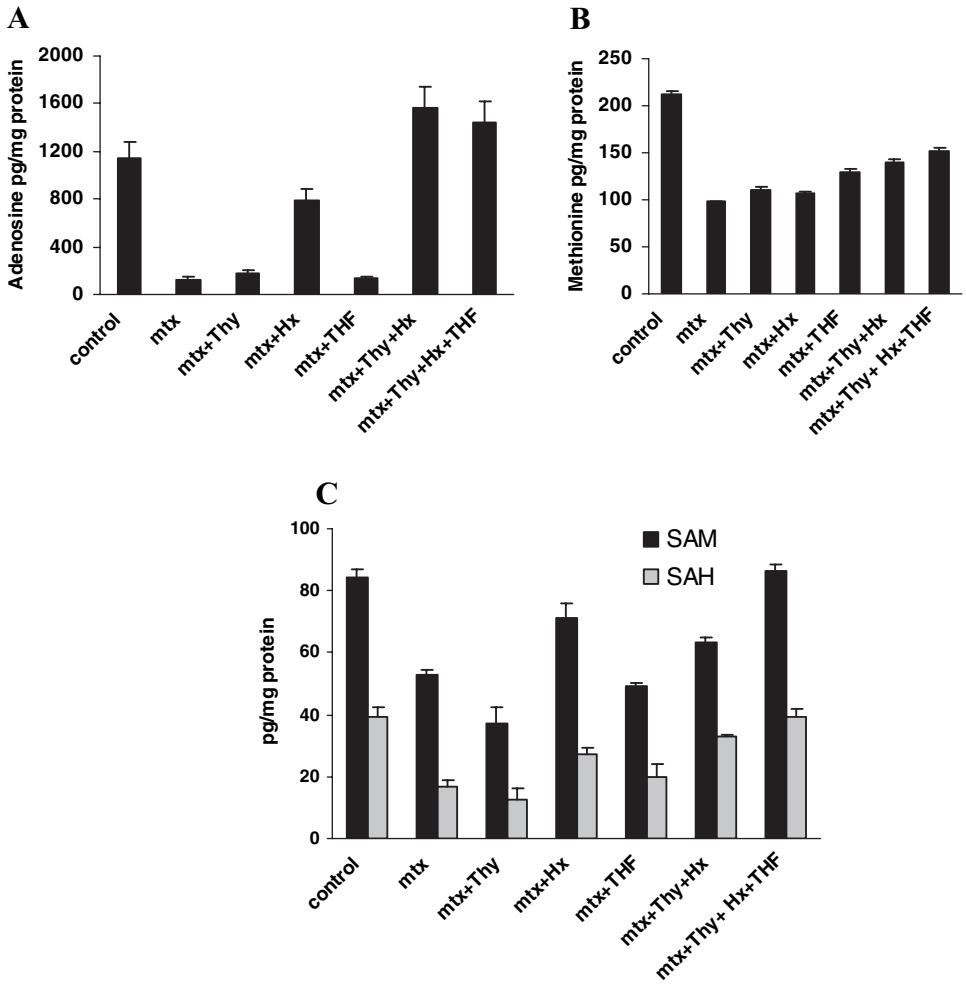

Fig. 3. LC-MS analysis of (**A**) adenosine (**B**) methionine and (**C**) S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). At 1 μM, MTX caused a significant decline in intracellular adenosine and methionine concentrations, and consequently reduced intracellular SAM and SAH concentrations.

properties or its ability to deplete intracellular adenosine we next sought to determine the effect of MTX on the expression of key enzymes involved in the methyl cycle and associated polyamine pathways by Western blot analysis. MTX treatment (1 μM) almost completely abolished the expression of methionine synthase (MS), an effect which was reversed by exogenous THF (50 μM) or a combination of thymidine and Hx (both at 100 μM) (Fig. 5A). Densitometric analysis revealed a 3-fold increase in MS expression relative to control in cells treated with MTX and THF suggesting that MTX-mediated decline in MS expression is due to folate deprivation. The expression of the other two enzymes involved in homocysteine metabolism (i.e., cystathionine β-synthase (Fig. 5D)) and S-adenosylhomocysteinase (Fig. 5B) was not affected by MTX. In contrast, MTX treatment (ranging from 1 to 4 μM) resulted in a non-dose dependent 2-fold upregulation of ornithine decarboxylase expression (Fig. 5C), the first enzyme in the polyamine pathway, as assessed by densitometry.

DISCUSSION

The novel findings from the present study are, firstly, that MTX induced differentiation of HT29 colorectal cells is mainly due its inhibitory actions on purine (i.e., adenosine) biosynthesis. The data also support a major role of cAMP, but rule out an involvement of PKC, in MTX-induced differentiation. In contrast, pyrimidine (i.e., thymidine) inhibition is unable to induce cellular differentiation. The present study also reveals the presence of active methyl cycle/transulfuration and polyamine pathways in HT29

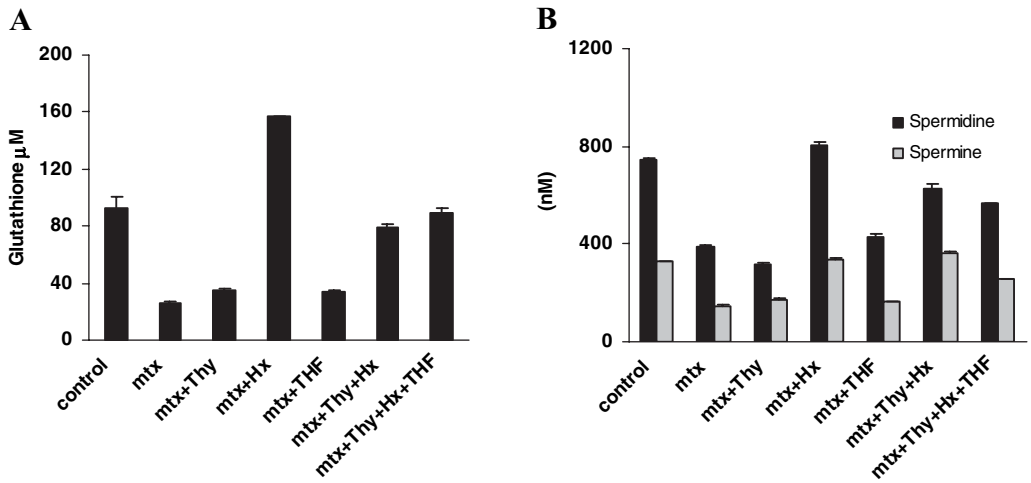

Fig. 4. HPLC analysis of (**A**) glutathione (GSH) and (**B**) polyamines. At 1 µM, MTX reduced intracellular GSH, spermidine and spermine concentrations; an effect reversed by Hx (100 µM) suggesting that the reduction in these metabolites is a consequence of the reduction in adenosine and consequently S-adenosylmethionine.

cells and demonstrates that treating these cells with MTX reduces intracellular SAM, SAH, polyamine and GSH concentrations, by depleting intracellular pools of adenosine.

Purines Versus Pyrimidines

The increase in MTX-induced ALP activity following supplementation with thymidine demonstrates that MTX-induced differentiation is not due to the inhibition of TS and, therefore, reduction in thymidine. This was further confirmed by the fact that 5-FU, a specific inhibitor of TS, did not increase ALP activity. Thus unlike keratinocytes, leukaemia cells and human choriocarcinoma cells, where the cytodifferentiating effects of MTX mainly arise as a result of thymine nucleotide depletion [Hatse et al., 1999], in HT29 human colon cancer cells it is apparently due to the deprivation of purines. Our data further show that co-administration of MTX with Hx or adenosine results in a complete inhibition of MTX-induced differentiation; thus confirming the pivotal role played by the inhibition of purine synthesis in MTX-induced differentiation.

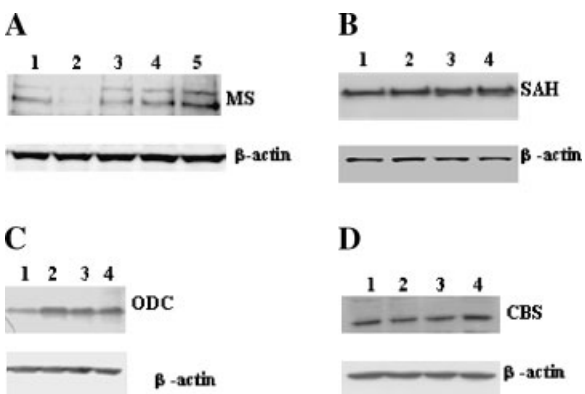

Fig. 5. Western blot analysis of (**A**) methionine synthase (MS) expression following treatment with 1 µM MTX (**lane 2**); 1 µM MTX plus 100 µM thymidine and Hx (**lane 3**); 1 µM MTX plus 100 µM thymidine and Hx, and THF (50 µM) (**lane 4**); and 1 µM MTX plus 50 µM THF (**lane 5**). **Lane 1** represents untreated control. Western blot analyses of (**B**) s-adenosylhomocysteinase, (**C**) ornithine decarboxylase and (**D**) cystathionine synthase following, in each case, treatment with 1 µM (**lane 2**), 2 µM (**lane 3**) and 4 µM (**lane 4**) MTX. **Lane 1** in each case represents untreated control. MTX downregulates MS and upregulates ornithine decarboxylase expression.

Cell Signaling

Since adenosine is required for cAMP synthesis we investigated if cAMP was involved in MTX-induced differentiation. Our results indicate that cAMP inhibition plays a central role in MTX-induced differentiation of colon cancer cells. This observation is supported by the very recent finding of Boucher et al. [2005] that cAMP/PKA signaling negatively regulates differentiation in Caco-2 colon cancer cells, suggesting a more general role of cAMP in intestinal cell differentiation. In contrast, the failure of staurosporine to induce ALP activity alone or to further enhance ALP expression in MTX treated cells (Fig. 3C) effectively eliminates the involvement of the PKC pathway in

MTX mediated cellular differentiation of HT29 colorectal cells. Since MTX is known to generally inhibit PI3-K/AKT and MEK-ERK signaling [Wang et al., 2001; Winter-Vann et al., 2003], we next assessed the effects of inhibiting these two pathways on cellular differentiation in HT29 cells. Our findings confirm a role for these signaling pathways in intestinal cell differentiation. However, the fact that both cell signaling inhibitors augmented MTX induced cellular differentiation suggests that MTX may either not fully operate via these pathways or that the dose of MTX used in the current study was insufficient to completely inhibit cell signaling via these pathways.

Cellular Metabolism

Although our study shows that THF supplementation to MTX treated cells upregulates MS protein expression beyond that of control cells, THF failed to reverse the growth inhibition caused by MTX; although it did inhibit MTX induced differentiation. At present we have no obvious explanation for this latter effect. Growth inhibition by low doses of MTX (nM ranges) can be reversed by THF [Kimura et al., 2004], suggesting that at these levels the antiproliferative effects of MTX are mainly due to the inhibition of dihydrofolate reductase. This was clearly not the case in the current study where the inhibition caused by the high dose of MTX was primarily due to the decline in pyrimidine and purine synthesis. Unlike low doses of MTX, which failed to reduce polyamine concentrations in adherent cells in the study of Kimura et al. [2004], our study showed a significant decline in polyamine concentrations at 1 μM MTX despite a 2-fold increase in the expression of ornithine decarboxylase. Polyamine concentrations were significantly greater in MTX treated cells supplemented with Hx compared to cells treated with MTX alone. This probably arose as a consequence of Hx supplementation resulting in greater intracellular concentrations of SAM, which is required for polyamine synthesis.

The decrease in intracellular SAM concentrations that accompanied MTX treatment appeared to be primarily due to the dramatic decline in intracellular adenosine concentrations. This observation is supported by the fact that the co-administration of MTX with Hx resulted in a significant increase in adenosine and SAM concentrations compared to cells treated with MTX alone; but this treatment had no effect on intracellular methionine concentrations suggesting that the reduction in intracellular methionine was non-limiting with respect to SAM synthesis in spite of a dramatic loss in expression of MS.

Winter-Vann et al. [2003] observed that MTX inhibited carboxylmethylation of Ras, causing its mislocalization to the cytosol and a 4-fold decrease in activation of p44 MAPK and AKT in mouse embryonic fibroblasts, and suggested that this effect was due to an accumulation of SAH. In HT29 cells, however, we observed a decrease in SAH and SAM concentrations, which was due to a sharp decline in intracellular adenosine. It is well documented that decrease in SAM limits the supply of methyl groups required for the synthesis of DNA, RNA and of aminopropyl groups for polyamine biosynthesis [Moinard et al., 2005]. Intracellular concentrations of SAM following MTX treatment were reduced to around 63% that of control cells. This reduction in SAM and polyamine concentrations, therefore, may have contributed to the decrease in cell proliferation and perhaps also to the induction of differentiation by MTX.

The data also show that the reduction in SAM concentrations in MTX treated cells led to a parallel decrease in intracellular GSH concentrations. This decline in GSH was not due to a reduction in cystathionine β-synthase expression, as determined by Western analysis, but probably to its activity, as SAM is a known positive regulator of cystathionine β-synthase activity [Janosik et al., 2001]. GSH depletion has been shown to be associated with the inhibition of cell cycle progression, increased cytotoxicity and induction of apoptosis by melphalan (L-phenylalanine mustard) in colorectal and pancreatic cancer cells [Vahrmeijer et al., 1999; Schnelldorfer et al., 2000]. Incubation of pancreatic adenocarcinoma cell line AsPC-1 with TGF-α resulted in enhanced cell proliferation whereas incubation of GSH depleted cells with TGF-α failed to stimulate proliferation [Schnelldorfer et al., 2000]. Thus MTX mediated GSH depletion may have contributed to the reduction in cell proliferation in the current study. GSH depletion has also been shown to augment butyrate induced differentiation in HT29 cells [Bernard and Balasubramanian, 1997]. The MTX mediated depletion of SAM and consequently GSH in the current

study could, therefore, have contributed to MTX-induced cellular differentiation.

Collectively, these results demonstrate a central role of purine deprivation in MTX induced cellular differentiation. Further investigations into the mechanisms involved in the regulation of cell differentiation will provide a better understanding of normal intestinal homeostasis, a better insight into the role of aberrations in these pathways in development of malignancy, and perhaps provide an alternative or complimentary approach to chemotherapy.

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