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ORIGINAL CONTRIBUTION

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Hyperproliferation of homocysteine-treated colon cancer cells is reversed by folate and 5-methyltetrahydrofolate

Summary *Background* There is an increasing evidence, stemming from epidemiological studies as well as studies performed in human biopsies and animal and cell culture models, suggesting that folate is chemopreventive in colonic carcinogenesis. Hyperhomocysteinemia is frequently associated with folate deficiency. Homocysteine, an amino acid, is metabolized to methionine in a 5-methyltetrahydrofolate (5-MTHF) dependent reaction. *Aim of the study* The aim of this study was i) to evaluate the effects of folate and its metabolites on growth and cell cycle progression in human colon cancer cells (Caco-2) in culture, and ii) to assess the effects of exogenous homocysteine on colon cancer cell proliferation. iii) Having found that homocysteine enhances colon cancer cell growth while metabolites of folate inhibit cell proliferation, we investigated the effects of simultaneous treatment in colon cancer cells. *Methods* Caco-2 cells were incubated either with homocysteine (0.1–10 μM), and/or with folic acid and its metabolites (0.625–10 μg/ml). Cell proliferation was determined after 24 h and 48 h by measuring 5-bromo-2'-desoxyuridine (BrdU) incorporation. Additionally, the cells were trypsinized and prepared for cell cycle determination using propidium iodide for DNA staining. The stained cells were analyzed using a flow cytometer. *Results* Folate inhibited cell proliferation moderately within 24 h. Its metabolites, dihydrofolate and 5-MTHF were more potent inhibitors of cell growth. Treatment with folate and 5-MTHF resulted in the accumulation of cells in G1-G0 phase of the cell cycle and decreased the number of cells in G2-M phase. In addition, cells treated with 5-MTHF were predominantly accumulated in the S-phase. There was no difference in cell cycle progression of Caco-2 cells treated with homocysteine in comparison to controls. In homocysteine-treated cells, both folate and 5-MTHF reversed the homocysteine-induced enhancement of growth. In contrast, folate reduced the Caco-2 cell growth rate to control values and 5-MTHF depleted growth of homocysteine-treated cells to levels significantly lower than controls. *Conclusions* Our data suggest that 5-MTHF, being the key metabolite in both the folate and homocysteine metabolic pathway, is the main modulator of growth-promoting actions of homocysteine as well as antiproliferative effects of folate in colon cancer cells.

Key words folate – colon cancer – homocysteine – chemoprevention – 5-methyltetrahydrofolate

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Introduction

There is an increasing evidence, stemming from epidemiological studies as well as studies performed in human biopsies, animal and cell culture models, suggesting that folate is chemopreventive in colonic carcinogenesis [1–4]. The underlying mechanisms of antiproliferative and chemopreventive actions of folate are not yet fully understood. However, the complexity of the folate metabolic pathway may suggest that different metabolites of folate might be involved in the multiple

EJN 446

European Journal of Nutrition (2004) Vol. 43, Number 2

effects of folate in normal, preneoplastic and malignant cells.

The folate metabolic pathway has been well elucidated. Folate is essential for the synthesis of S-adenosylmethionine, and is thereby involved in DNA methylation. Furthermore, folate is metabolized (via dihydrofolate reductase) to dihydrofolate and tetrahydrofolate, and reduced to 5-methyltetrahydrofolate via 5,10-methylenetetrahydrofolate through 5,10-methylenetetrahydrofolate reductase (MTHFR). 5,10-Methylenetetrahydrofolate is a product of tetrahydrofolate [5]. 5-Methyltetrahydrofolate (5-MTHF) serves as a methyl donor during the remethylation of homocysteine to methionine, which is in turn converted to S-adenosylmethionine (SAM). SAM methylates specific cytosines in the DNA and is therefore able to regulate gene transcription. As a consequence of folate deficiency, cellular SAM is depleted, which in turn reduces DNA methylation and may induce the expression of protooncogenes, therefore resulting in cancer [6]. Furthermore, folate is an important factor for de novo biosynthesis of purines and thymidylate, which are essential for DNA synthesis and repair. It is therefore not surprising that in vivo and in vitro studies support the evidence that folate is essential in the maintenance of DNA integrity and stability. Animal studies indicated further a cause-and-effect relationship between folate deficiency and colorectal carcinogenesis, as well as the dose-dependent protective effect of dietary folate supplementation even if it is just above the basal dietary requirement [8]. A common mutation (677C → T) in MTHFR was found to reduce colon cancer risk in humans with normal plasma folate levels, probably by increasing 5,10-methylenetetrahydrofolate levels for DNA synthesis. As a result, folate or its metabolites may act by increasing DNA methylation, and by repairing and reducing formation of DNA strand breaks of p53 and Apc genes [6–8].

Hyperhomocysteinemia is frequently associated with folate deficiency. Homocysteine is an amino acid and a product of methionine metabolism, and a precursor of methionine synthesis. Intracellular homocysteine is rapidly secreted from the cell. It has been long postulated that the plasma homocysteine concentration is inversely related to the occurrence of cardiovascular and cerebrovascular diseases [9–13]. More recently, increased homocysteine concentrations in plasma have been attributed as a risk factor for cancer and even as a new tumor marker [14].

The aim of this study was i) to evaluate the effects of folate and its metabolites on growth and cell cycle progression in human colon cancer cells (Caco-2) in culture and ii) to assess the effects of exogenous homocysteine on colon cancer cell proliferation. Having found that homocysteine enhances colon cancer cell growth while metabolites of folate inhibit cell proliferation, we investigated the effects of simultaneous treatment in colon cancer cells.

Materials and methods

Chemicals

Homocysteine, folic acid (FA), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), 5-methyl-tetrahydrofolic acid (5-MTHF) and methotrexate (MTX) were obtained from Sigma (Deisenhofen, Germany). Cell culture media and supplements were from GIBCO BRL (Eggenstein, Germany), antibiotics used for cell culture were from Biochrom (Berlin, Germany) and cell culture plates were from Nunc (Roskilde, Denmark).

Cell culture

Caco-2 cells were obtained from American Type culture collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml) at 37 °C in an atmosphere of 95 % air and 5 % CO_2. According to the manufacturer's information, the amount of folic acid in DMEM was 4 µg/ml. For the experiments, 5000 cells per well were seeded on a 96 microtiter plate and incubated either with folic acid and its metabolites (0.625–10 µg/ml) and/or with supplemental homocysteine (0.1–10 µM).

Cell proliferation and cell death

Cell morphology was routinely assessed by phase contrast microscopy, and cell viability was evaluated by i) estimating the percentage of attached cells, and ii) by staining the cells with trypan blue solution and estimating a number of trypan blue-positive cells; dead cell count in culture was considered as normal (i. e., not suggestive of cell death) if not exceeding 5 % of the total cell number. Cell proliferation was determined after 24 h and 48 h by measuring 5-bromo-2'-desoxyuridine (BrdU) incorporation, using a commercially available assay kit (Boehringer Mannheim, Germany). To drive the cells through the cycle completely, in selected experiments cell growth was evaluated by counting in a hemocytometer.

Cell death was evaluated by FACS after staining with propidium iodide, as described previously [15]. For statistical significance, the t-test was performed with Sigma-Stat (San Rafael, CA).

Cell cycle measurement

The cells were seeded on 6-well plates at a density of 250,000 cells per well. The cells were then incubated either with homocysteine (0.1–10 µM) or folate and its

metabolites at concentrations of 1 µg/ml and 10 µg/ml. At 24 and 48 h after the incubation had been initiated, the cells were trypsinized and prepared for cell cycle determination using propidium iodide for DNA staining. The stained cells were analyzed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) with CellQuest Software. The histograms were then evaluated with ModFit LT2.0 (Verity Software, VA, USA).

Results

Effect of folate on DNA synthesis in Caco-2 cells was only negligible within the first 24 h in culture, while its metabolites, dihydrofolate and 5-MTHF, were more potent inhibitors of cell growth (Fig. 1). After 48 h of treatment, even at low concentrations, the effect of folate became more prominent, indicating that folate might be metabolized before exerting its maximal effects on cell proliferation. Tetrahydrofolate significantly increased DNA synthesis at the highest concentration after 24 h, but this effect was not maintained after 48 h in culture (Fig. 1).

Within 48 h of treatment, there was no increase in cell detachment, viability or apoptosis in the cells exposed to either folate or its metabolites (data not shown). Both in control and treated Caco-2 cells, the percentage of apoptotic cells did not exceed 5 %.

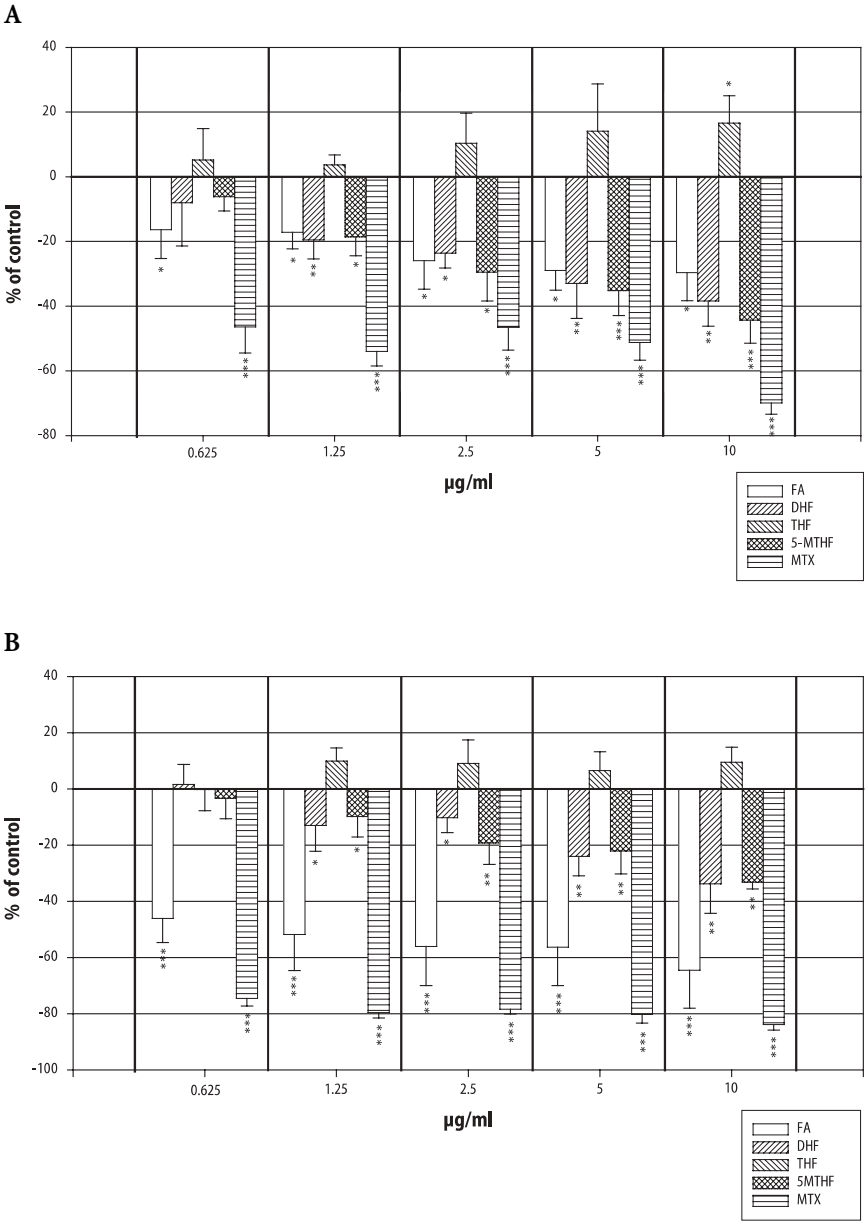

Fig. 1 Effects of folate and its metabolites (dihydrofolate; tetrahydrofolate; and 5-methyl tetrahydrofolate) on proliferation of human colon cancer cells (Caco-2), after 24 (**A**) and 48 h (**B**) of treatment. The cells were allowed to attach overnight and then treated with increasing concentrations of folate or the metabolites. Methotrexate (MTX) was used as positive control. Proliferation was measured by BrdU incorporation. Mean ± SD; n = 8; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

European Journal of Nutrition (2004) Vol. 43, Number 2

In contrast to folate and its metabolites, homocysteine stimulated proliferation of Caco-2 cells after both 24 and 48 h in a dose-dependent manner, with peak effect at concentrations between 1 and 2 µmol/L (Fig. 2). There was again no change in cell viability and apoptosis, as assessed by trypan blue incorporation and FACS (data not shown).

Cell cycle analysis was performed after both 24 and 48 h of treating the cells with homocysteine and folate and its metabolites. Over the initial 24 h, treatment with folate and 5-MTHF resulted in the accumulation of cells in G1-G0 phase, and a decrease in the number of cells in G2-M phase compared to controls. The cells treated with 5-MTHF were additionally accumulated in the S phase. There was no significant difference in cell cycle progression of Caco-2 cells treated with homocysteine and controls (Fig. 3).

We subsequently measured the effect of folate, 5-MTHF and homocysteine on Caco-2 cell proliferation after 24 h of treatment. At this time point, homocysteine was a potent inducer of cell growth. At concentration of 10 µg/ml, folate decreased the cell proliferation rate moderately but significantly, while 5-MTHF was a more prominent inhibitor of Caco-2 cell proliferation than folate itself (Fig. 4). In homocysteine-treated cells incubated with folate or 5-MTHF, both folate and its metabolite were able to inhibit homocysteine-induced enhancement of growth. While folate reduced the Caco-2 cell growth rate to control values, 5-MTHF depleted growth of homocysteine-treated cells to the levels significantly lower than controls.

Discussion

Emerging evidence indicates that folate modulates the risk of developing colorectal cancer: folate depletion is suspected to enhance carcinogenesis, and folate supplementation is thought to be protective against cancer. Because folate has a critical role in both DNA methylation and DNA synthesis, most studies have focused on these two actions. Folate depletion has been found to change DNA methylation both in animal and human studies. Impaired uracil incorporation into DNA, reflecting an imbalance of nucleotide synthesis, has also been observed in human studies studying folate-depleted subjects. Both critical conditions could be reversed by folate supplementation [4–6].

Our data imply, first, that homocysteine does enhance growth of colon cancer cells in culture. Second, the growth-promoting effect of homocysteine was reversed by folate, while 5-methyltetrahydrofolate (5-MTHF), a product of folate metabolization by methyl tetrahydrofolate reductase, inhibited homocysteine-stimulated growth below the control values. Incubation with 5-MTHF resulted in an accumulation of the cells in the S-phase. Together with our preliminary study [15], these data imply that 5-MTHF may be the active compound in growth-inhibitory actions of folate in colon cancer cells. This metabolite, being a co-factor in S-adenosylmethionine synthesis (occurring during the S-phase of the cell cycle and influencing DNA methylation) may be the metabolic turning point of the antiproliferative effects of folate in colon cancer cells.

The basal concentration of folic acid in the cell culture medium used in these experiments was 4 µg/ml,

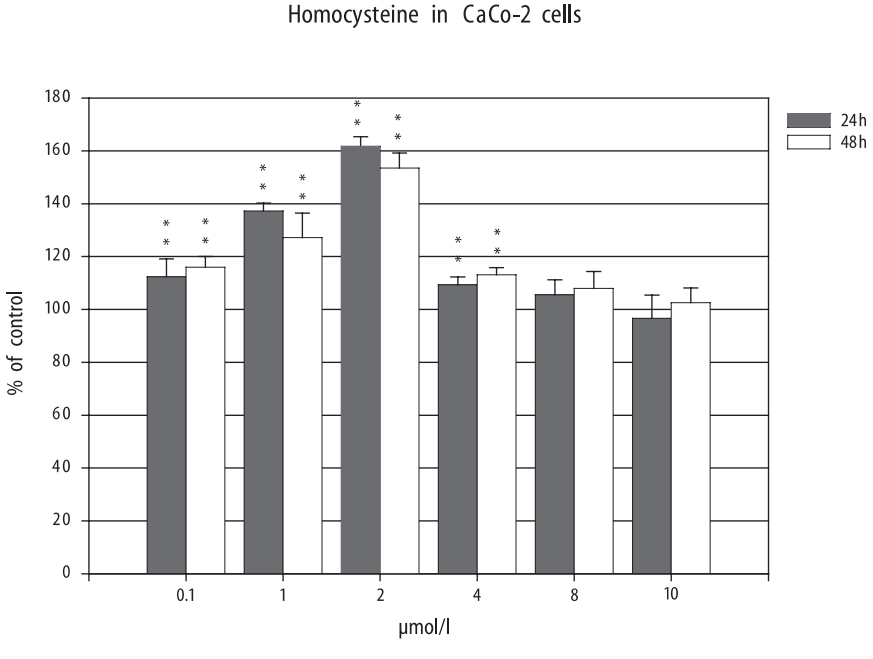

Fig. 2 Effect of homocysteine on Caco-2 cell growth. The cells were allowed to attach overnight and then treated with increasing concentrations of homocysteine. Proliferation was measured by BrdU incorporation. Mean ± SD; n = 8; ** $P < 0.01$

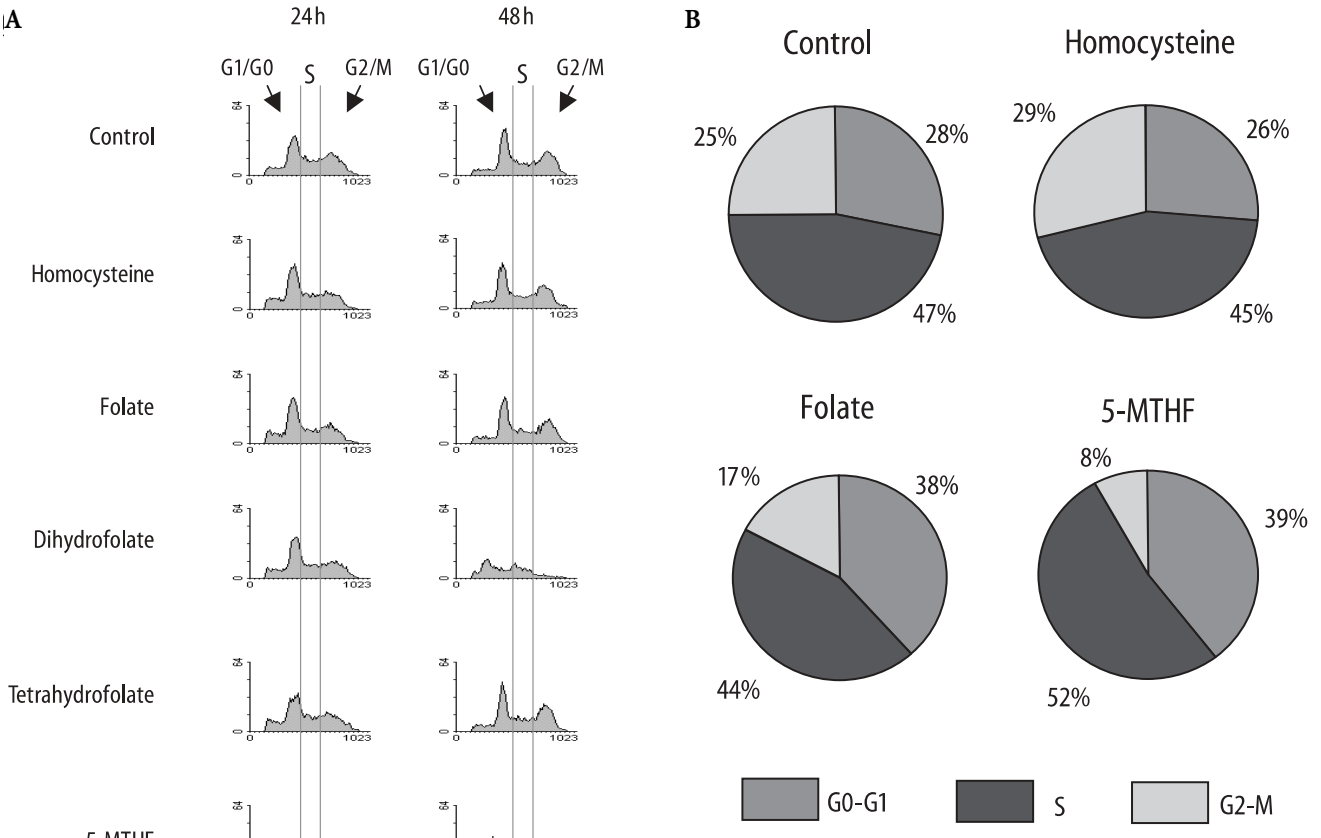

Fig. 3 **A** Representative cell cycle histograms (DNA content vs. events, G1/G0-, S- and G2/M-Phase) of Caco-2 cells treated with folate and its metabolites (10 µg/ml, 24 and 48 h). **B** Cell cycle distribution in Caco-2 cells treated with homocysteine, folate and 5-MTHF. Treatment with folate and 5-MTHF resulted in the accumulation of cells in G1-G0, and S-phase and a decrease in the number of cells in G2-M

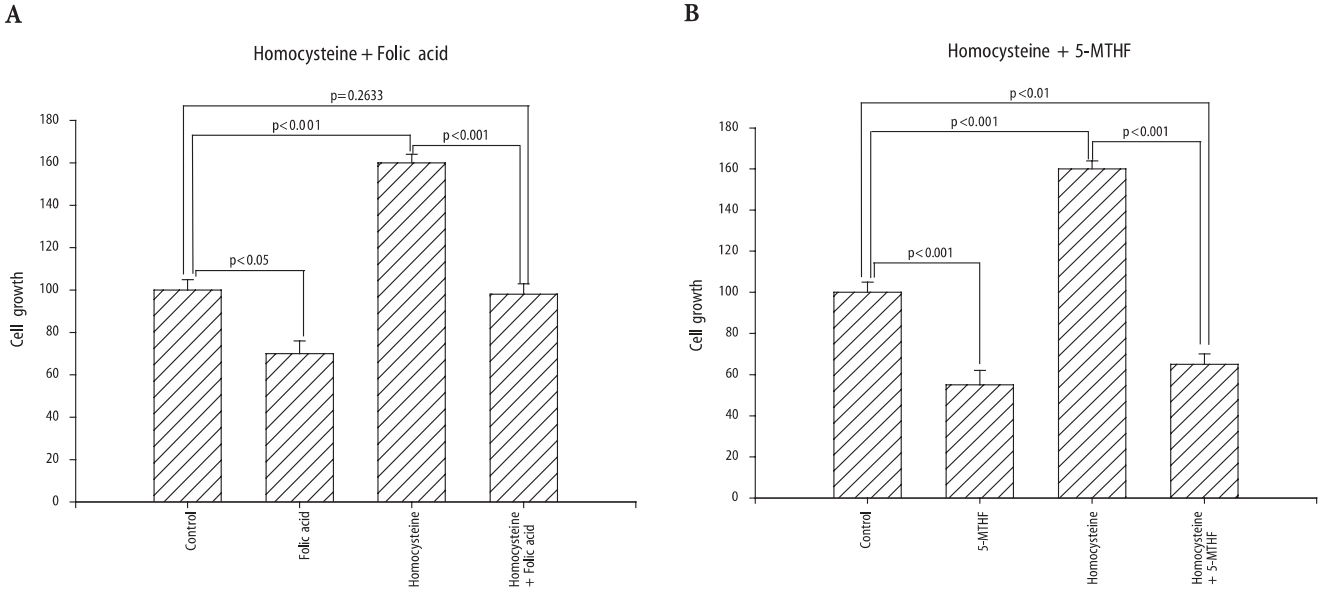

Fig. 4 Folate (**A**) and 5-MTHF (**B**) inhibit homocysteine-induced hyperproliferation of Caco-2 cells. The cells were treated with homocysteine (2 µmol/L) with or without folate or 5-MTHF (10 µg/ml) for 24 h. Proliferation was measured by BrdU incorporation. Mean ± SD; n = 8

which is already higher than physiological levels of folate described in blood and intestinal and colonic lumen. However, we used Caco-2 cells as an experimental model. These cells originate from human colonic adenocarcinoma, and, in culture, resemble true cancer cells as well as immature colonocytes [16]. Because of all peculiarities that may occur when cells in culture are used to test growth-related effects (for example, the substance-to-cell relationship [17]), the concentrations of the investigated substances *in vitro* are usually far above those occurring in vivo.

The growth-stimulating effect of homocysteine was reversed by folate, but 5-MTHF was more efficient in reversing the homocysteine-mediated growth in Caco-2 cells. Homocysteine can enhance growth of not only neoplastic cells. For example, both in vitro and in vivo, homocysteine stimulated cardiomyocyte proliferation [10, 18]. Another important pathophysiological effect of homocysteine is oxidative modulation of the endothelial membrane of blood vessels [19, 20]. In vitro, homocysteine at concentrations equivalent to those described in hyperhomocysteinemia in humans, significantly promoted both human and pig smooth muscle cell proliferation, while inhibiting endothelial cell growth [21]. In our study, we evaluated only direct effects of homocysteine on cells in culture. Whether additional effects are seen when the entire organ system is studied still remains to be seen.

The likely explanation of the mechanisms involved in the antiproliferative actions of 5-MTHF may, at least in part, lie in its key roles in both folate and homocysteine metabolic pathways. Remethylation due to the activity of the enzyme methionine synthase is the major event in the conversion of homocysteine to methionine by adding a methyl group to the homocysteine molecule [22]. The enzyme methionine synthase requires 5-MTHF as a methyl donor. This metabolic pathway occurs in all organs; only in the liver is the conversion of homocysteine to methionine catalyzed by betaine-homocysteine methyl transferase [23].

Hyperhomocysteinemia, which is indicative for an impairment of one-carbon metabolism, has been regarded as an important risk factor for cardiovascular disease. Its likely role in carcinogenesis has been shown in only a few studies so far. Kato and colleagues [24] reported that the risk of colorectal cancer was significantly elevated in patients with high total plasma homocysteine concentrations. With multivariate analysis, there was a strong trend toward an effect of total plasma homocysteine independently from folate concentrations. Weinstein et al. [25] showed that invasive cervical cancer risk is significantly increased in women with hyperhomocysteinemia.

Our data suggest that 5-MTHF, being the key metabolite in both folate and homocysteine metabolic pathway, might be the main modulator of growth promoting actions of homocysteine as well as antiproliferative effects of folate in colon cancer cells. Cellular availability of 5-MTHF may be of great importance in regulating cellular effects of homocysteine related to cell growth.

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