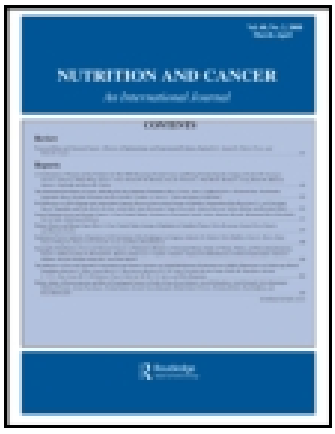

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Folic acid (FA) consumption at high levels has been associated with colon cancer risk. Several mechanisms have been proposed to explain this association. The Notch signal pathway has been implicated in the regulation of cellular proliferation. Our aim was to demonstrate that high concentrations of FA or its reduced form, 5-methyltetrahydrofolic acid (5-MTHF), increase colorectal carcinoma HT29 cell proliferation through an increase of Notch1 activation and to prove if the inhibition of Notch1 activation by gamma secretase inhibitor, reduce the effect of folic acid. HT29 cells were cultured in high (400 nM), low (20 nM), or 0 nM FA or 5-MTHF concentrations during 96 h with or without DAPT (gamma secretase inhibitor). Cell proliferation was determined by the methylthiazole tetrazolium method, and Notch1-intracellular domain (NICD) was analyzed by flow cytometry. HT29 cells exposed to 400 nM FA or 5-MTHF showed higher proliferation rate than those exposed to 20 nM of FA or 5-MTHF ($P < 0.01$) during 96 h. NICD expression increased at higher FA or 5-MTHF concentrations compared with lower concentrations ($P < 0.01$). This effect on proliferation was partially reversible when we blocked Notch1 activation with the inhibitor of γ-secretase ($P < 0.05$).These data suggest that high concentration of FA and 5-MTHF induce HT29 cell proliferation activating Notch1 pathway.

INTRODUCTION

Folate plays an essential physiological role in 1-carbon metabolism. It is required for the de novo synthesis of purines and pyrimidines, essential elements for the de novo production of RNA and DNA, and for the synthesis of S-adenosyl-methionine, which is required for methylation of DNA, histones, lipids, and neurotransmitters (1, 2). Folate deficiency in the periconceptional period is associated with neural tube defects. In the brain of animal models, folate promotes proliferation of neural stem cells and its depletion inhibits their proliferation (1, 2). For this reason, several countries, including Chile, have started successful food folic acid fortification programs to cover the requirements (400 μg) of the target population. In many countries, neural tube defects dropped by 25% to 50%, since the addition of folic acid to grain-based food or to wheat flour (3–5).

However in 20% to 37% of the general population, serum folate levels reaches values above 40 nmol/l after fortification, considered as supraphysiologic levels (6, 7). There is an ongoing discussion about the possible adverse effects of folic acid intake from fortified products, motivated by conflicting reports about heart disease risk, masking of vitamin B12 deficiency and an increasing risk of cancer (6, 8, 9). Several mechanisms have been proposed to explain the association between folate and cancer, including modulation of cancer cell replication and derangements in global and gene-specific methylation status. (10). The importance of folate analogues (e.g., methotrexate) for cancer treatment provides strong evidence that folate is also required by rapidly proliferating tumor cells. Indeed, treatment with high doses of folate (4–20 times basal needs) in mouse models of colon carcinoma and in clinical studies of pediatric populations with leukemia, resulted in worsening of cancer (11–13). Also epidemiological studies have shown a temporal association between colon cancer prevalence and folic acid fortification (14–16).

Folate receptors (FRs) are a family of membrane-anchored glycoproteins of 32–36 kDa with a very high affinity for folic acid and N^5-substituted reduced folates. They are involved in folate cellular uptake and homeostasis. At least 3 distinct FR isoforms, FRα, β, and γ, have been cloned from a variety of cell lines and tissues. FRα and β show different affinities for

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folate co-enzymes and antifolate drugs and are differentially regulated in several tissues. High levels of FRα have been observed in malignant tissues of epithelial origin, particularly in ovarian carcinomas, whereas very low levels have been found in normal tissues (17). Moreover, different investigators have reported that cells cultured under restricted folate conditions acquired growth advantages when they were transfected with the FRα gene (18). However there are few data about the effect of folate on the regulation of FRα.

On the other hand, in animal models and in vitro studies, folic acid supplementation promotes proliferation and inhibits differentiation of embryonic neurologic stem cells by activating Notch signaling, preventing malformations that appear during embryogenesis in the neural system (19, 20). The Notch signaling pathway drives proliferation, differentiation, apoptosis, cell fate, and maintenance of stem cells in several proliferating tissues. This signaling pathway is necessary for cell–cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life (21). Notch signaling is upregulated in different types of cancer such as T-cell acute lymphoblastic leukemia (22), ovarian (23), breast (24), nonsmall cell lung (25), and colon cancer (26). Moreover, Notch1 inhibition prompts apoptosis and retards tumor cell proliferation in the above mentioned cancers (27).

On the contrary, Notch1 upregulation acts as a tumor suppressor in keratinocytes of the epidermis, human esophageal squamous cell carcinoma cell line EC9706 (28), human medullary thyroid cancer (29), cervical cancer cell (30), and pheochromocytoma and carcinoid tumors (31). These evidences suggest that the effects of Notch1 signaling pathway are cell context-specific. Consequently, folate could be a key nutrient in regulating cell growth/proliferation, through Notch1 activation, preventing neural tube defects during embryogenesis and promoting specific cancer cell proliferation in adult life.

The aim of this study was to assess the in vitro effect of low and high concentrations of either 5-MTHF or folic acid on cell proliferation, FRα distribution, and Notch 1 signaling activation in human colorectal carcinoma HT-29 cell line.

METHODS

Cell Culture

HT-29 (human colon adenocarcinoma grade II) cell line was cultured in folate free-RPMI 1640 containing 10% dialyzed fetal bovine serum (Gibco, California), 2 mM glutamine, and 50 U/mL penicillin-streptomycin. Cells were maintained at 37°C under humidified conditions and 5% CO2. To establish experimental conditions under folate treatment or γ-secretase inhibitor (DAPT) (Calbiochem, Darmstadt, Germany), to inhibit Notch signaling, cells (5 ċ 104) were cultured in 24-well culture plates with either folic acid or 5-MTHF (20 and 400 μM), DAPT (100 μM), or dimethyl sulfoxide (DMSO) as a control. Cells were harvested at indicated time points and proliferation assays as well as folate receptor α and Notch intracellular domain (NICD) quantification were performed. Cell culture viability was assessed by trypan blue exclusion test.

Cell Proliferation Study (Methylthiazole Tetrazolium Assay)

HT-29 cell proliferation was assayed every 24 h up to 96 h of culture time using the methylthiazole tetrazolium (MTT) Cell titer 96 AQueous (Promega, Wisconsin) kit according to the manufacturer's instructions. Briefly, 20 μL of MTT solution was added to each well. Cells were then incubated at 37°C for a further 4 h. The absorbance of the purple formazan, reduction product of MTT by viable cells, was measured at 490 nm. All experiments were performed in triplicate and repeated 3 times.

Flow Cytometry

To quantitate the cell surface folate receptor α (FRα), 1 ċ 106 HT-29 cells were harvested after 72 h of culture with folate and washed twice with PBS. Cells were then stained with primary antibody antihuman α-folic receptor 1 (R&D Systems, Minnesota), and incubated for 45 min at 37°C. Afterwards, cells were rinsed twice with PBS to remove antibody excess and further incubated with secondary antigoat-PE conjugated antibody (R&D systems) during 15 min at 37°C. After rinsing twice with PBS, fluorescence was measured in a Flow Cytometer (FACSORT BD Biosciences, San Jose, CA). To further quantitate the amount of cell surface and internalized FRα, cells were additionally suspended and incubated overnight in a solution of PBS, 70% ethanol, 10% fetal bovine serum. Then, cells were washed twice with PBS and FRα was stained and measured as described above.

To assess NICD, 1 ċ 106 HT-29 cells were suspended as described above. Cells were stained with NICD-PE conjugated antibody (Becton and Dickinson) and incubated at 37°C during 30 min. Cells were then rinsed twice with PBS to eliminate antibody excess and fluorescence was measured in a Flow Cytometer (FACSORT BD Biosciences, San Jose, CA).

Statistical Analysis

Results were expressed as the mean value $\pm$ SD. The significance of difference was determined by analysis of variance for repeated measured, with $P < 0.05$ regarded as statistically significant.

RESULTS

HT-29 Cell Proliferation

HT-29 cell proliferation rate until 96 h of culture was significantly higher in RPMI-1640 medium containing high

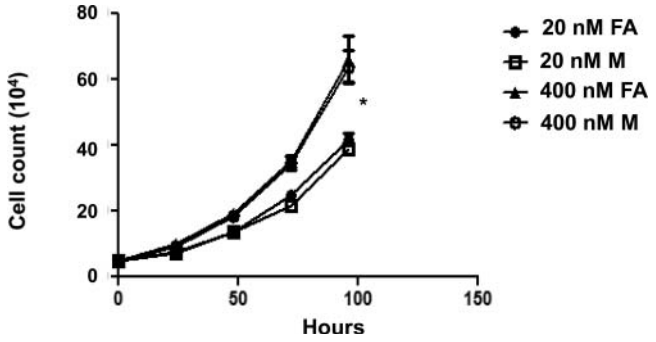

FIG. 1. Effect of folic acid (FA) and 5-methyltetrahydrofolic acid (M) on HT29 cell proliferation. *$P < 0.001$.

($400\ \mu$M) concentrations of either folic acid or 5MTHF than cells cultured with low concentrations ($20\ \mu$M) of both folic acid or 5MTHF ($P < 0.001$) (Fig. 1).

FRα1

After 96 h of cell culture in the presence of either folic acid or 5MTHF at $400\ \mu$M, in conditions where HT-29 cell proliferation was enhanced by folates, the membrane associated FRα1 decreased by approximately 25%. Moreover, in the presence of low folate concentrations ($20\ \mu$M), no change in membrane associated FRα1 was observed (Figure 2A). However total cellular FRα remained unchanged (Fig. 2B).

NICD

To investigate whether Notch signaling was involved in folates-stimulated HT-29 cell proliferation, we next quantified the levels of NICD on HT-29 cells cultured for 96 h at high ($400\ \mu$M) and low ($20\ \mu$M) concentrations of both folic acid and 5MTHF. As shown in Fig. 3, NICD levels increased approximately 20% after 96 h of cell culture with high concentrations ($400\ \mu$M) of either folic acid or 5MTHF ($P <$ 0.05). At low concentrations of both folates, no change was observed in intracellular levels of NICD.

y-Secretase Inhibitor (DAPT)

To examine the role of Notch signaling in the folate-stimulated HT-29 cell proliferation, we cultured HT-29 cells for 96 h at high ($400\ \mu$M) and low ($20\ \mu$M) concentrations of both folic acid and 5MTHF with or without DAPT. The results showed that DAPT had a significantly inhibitory effect on cell proliferation cultured with high concentrations of either folic acid or 5MTHF. This inhibitory effect of DAPT was not observed among cells cultured with low concentrations of either folic acid or 5MTHF (Fig. 4). There were no significant differences in cell viability between treated and untreated cells at all-time points as measured by trypan blue exclusion.

DISCUSSION

In this study we show for the first time that proliferation of HT-29 cells, a human colon cancer cell line, is enhanced by high concentrations of either folic acid or 5MTHF. Moreover, we observed that this effect is dependent on the activation of Notch1 signaling.

Folates at a concentration of $400\ \mu$M, which could be considered supraphysiological, stimulated the rate of proliferation of HT-29 in a time-dependent manner exceeding the rate of proliferation exhibited by HT-29 cells cultured with $20\ \mu$M folates (physiological levels). This effect is in accordance with the fact that antifolate drugs such as methotrexate are used as a treatment for rapidly proliferating tumor cells. Three mechanisms of membrane transport of folates into cells and across epithelia have been described, namely reduced folate carrier, Folate receptor (FRα, β, δ, and the soluble form γ) with a high affinity for folic acid and 5MTHF and the Proton-coupled folate transporter (32, 33). FRα levels are elevated in specific malignant tumors of epithelial origin (34). Interestingly in this study, a significant reduction in membrane associated-FRα

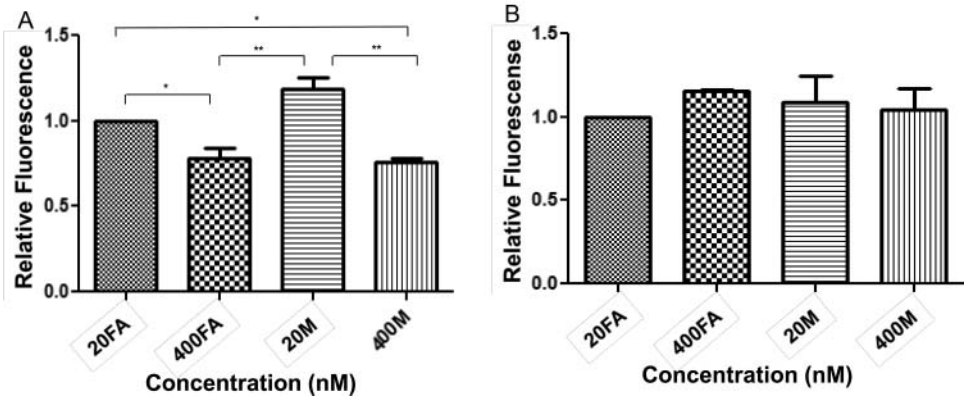

FIG. 2. A: External folate receptor α1 (FRα1) expression of HT29 cell at 20 M and 400 M of folic acid (FA) or 5-methyltetrahydrofolic acid (M) exposure. B: Total folate receptor α1 (TFRα1) expression of HT29 cell at 20 M and 400 M of FA or 5-methyltetrahydrofolic acid exposure. *$P < 0.05$. **$P < 0.01$.

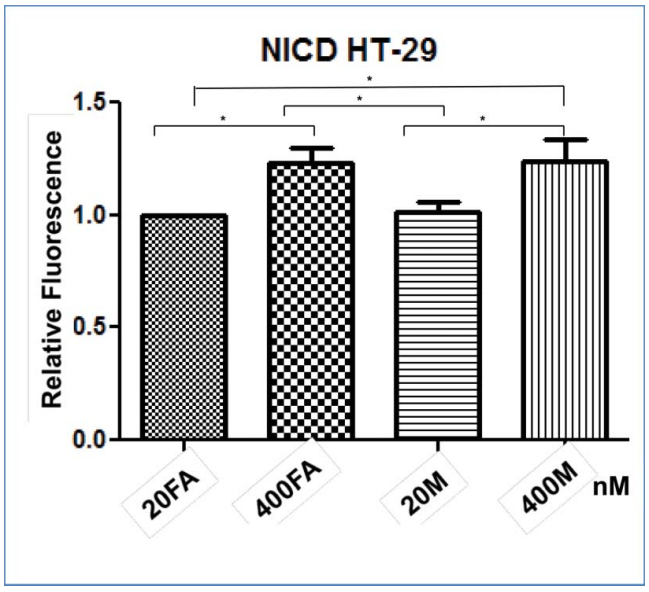

FIG. 3. Notch-1 Intracellular domain (NICD) expression of HT29 cell at 20 M and 400 M of folic acid (FA) or 5-methyltetrahydrofolic acid (M) exposure. *$P < 0.05$.

bioavailability was observed, when HT-29 cells were cultured in folic acid-free media supplemented with high concentrations of either folic acid or 5MTHF. Considering that total cellular FRα remained unchanged, it is unlikely that the higher extracellular folate concentration affected FRα gene expression. Therefore, it is plausible that the excess of folate in the culture media modified FRα recycling of HT-29 cells. FRα quantitatively recycles between the cell surface and intracellular compartments. In fact, the recycling FR usually shows a 1:1 distribution between the cell surface and endocytic compartments in confluent cells (35, 36). Thus, it is probable that, in our conditions, the high extracellular concentrations of folates effectively forced the internalization of receptor-bound folate modifying the ratio between the membrane associated and intracellular FRα. Although we did not measure intracellular levels of folates we can propose that the enhanced folate uptake from the cell culture media leads to increased intracellular levels of folate, facilitating rapid cellular growth and division of the HT-29 cell line. FRα might affect cell proliferation through several mechanisms such as nucleotide synthesis, nucleic acid methylation, or by generating regulatory signals (37, 38).

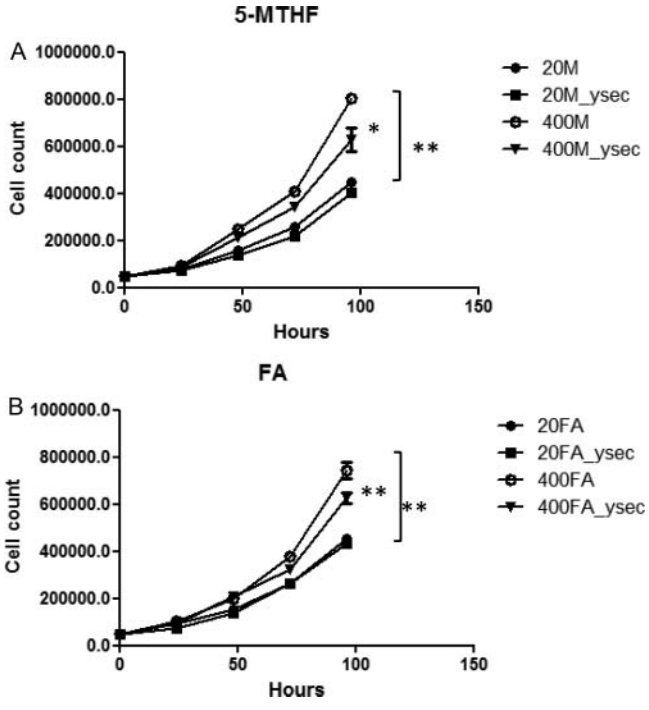

FIG. 4. Effect of 5-methyltetrahydrofolic acid (M) (A) and folic acid (FA) (B) on HT29 cell proliferation with and without inactivation of Notch signaling by the use of γ-secretase inhibitor (DAPT). * $P < 0.05$. **$P < 0.01$.

Accumulating data indicates that tumor formation might result from the deregulation of numerous signaling pathways such as Notch, Wnt/β-catenin pathway and SHH pathways (39, 40). Our results show that high extracellular levels of folates induced a significant rise in Notch intracellular domain of HT-29 cells. These data suggest a possible role of Notch signaling in the induction of HT-29 colon cancer cell proliferation by high folate concentrations. These data are in accordance with previous published data by Moreno et al. that suggest that FRα participates in pituitary tumor formation, and this effect may in part be due through Notch3 signaling regulation (41). Moreover, our results are consistent with recent findings showing that colon cancer progression is mediated by high levels of Jagged1/Notch signaling (41).

To further investigate the effect of Notch activation on high folate-mediated HT-29 proliferation, we treated HT-29 cultured cells at high concentrations of folates with DAPT, which inhibits γ-secretase activity and is a crucial activation step of Notch signaling. The inhibitor induced a significant reduction in the rate of HT-29 proliferation. However, this proliferation rate was higher than that of HT-29 cells cultured at low concentrations of folates. These results are in accordance with other authors, who demonstrated that the inhibition of γ-secretase slows but does not suppress HT29 proliferation in normal culture conditions (42). Therefore, Notch signaling should be one but not the only mechanism involved in the enhanced rate of proliferation exhibited by HT-29 cell in the presence of high concentration of folates.

There are reports showing that γ-secretase inhibitors (GSIs), which block Notch activation, are a suitable tool for treating human cancers. In advanced or metastatic thyroid cancer, nonsmall cell lung cancer, intracranial tumors, sarcoma or desmoid tumors, colorectal cancer with neuroendocrine features, melanoma and ovarian cancer, GSIs as single agents exhibit antitumor activity. The side effects of these inhibitors, especially in the gastrointestinal tract, are a powerful limitation for their clinical use at this moment (43, 44).

In conclusion, our study shows for the first time that high concentrations of either folic acid or 5MTHF stimulate HT-29 cell proliferation in a Notch signaling dependent manner. Our findings suggest that supraphysiological levels of folates may promote tumorigenesis in human colorectal cancers trough activation of Notch signaling.

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