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Progesterone receptor activation is required for folic acid-induced anti-proliferation in colorectal cancer cell lines

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ABSTRACT

Previously, we demonstrated that folic acid (FA) could inhibit proliferation of colorectal cancer cell lines through activating the folate receptor (FR)α/cSrc/ERK1/2/NFκB/p53 pathway and anti-COLO-205 tumor growth *in vivo*. Since we recently also demonstrated that female sex hormones could affect the FA's action in regulating endothelial cell proliferation and migration, the aim of this study was to investigate the effect of progesterone (P4) on the FA-induced anti-proliferation in colorectal cancer cells. Treatment with FA significantly reduced the proliferation of the P4 receptor (PR)-positive colon cancer cell lines, COLO-205, HT-29 and LoVo, but did not significantly affect the proliferation of the PR-negative colon cancer cell lines, HCT116 and DLD-1. Pre-treatment with Org 31710, a PR specific antagonist, abolished the FA-induced proliferation inhibition and activation in the signaling pathway involved in regulating proliferation inhibition in these PR positive colorectal cancer cell lines. The involvement of PR in the FA-induced activation of cSrc and up-regulations in cell cycle inhibitory proteins (p21, p27 and p53) was confirmed by knock-down of PR expression using the siRNA technique. Importantly, we show direct protein interaction between FR and PR in COLO-205. Moreover, treatment with FA induced PR activation in COLO-205. Taken together, these data suggest that FA induced proliferation inhibition in colon cancer cells through activation of PR. This finding might explain some of the controversies of FA's effects on cancer growth and provide valuable reference for clinical applications of FA in treating colorectal cancer.

Introduction

Colorectal cancer is a major cause of morbidity and mortality throughout the world. Currently, it is the third most common cancer worldwide and the fourth most common cause of death. Although it has been indicated that incidence rates for colon cancer in both sexes are similar, accumulating evidence has suggested that sex hormones are relevant to the development of colorectal cancer. Epidemiological studies in women have shown that increases in female hormones such as estrogens and progestin are associated with a lower risk for developing colorectal cancer [1–3]. In support of these results, reports from the Women's Health Initiative trial showed an approximately 40% lower risk for colorectal cancer in the estrogen plus P4 group as compared with the placebo group [4,5]. However, the other Women's Health Initiative trial did not show a lower risk of colorectal cancer among hysterectomized women treated with estrogen alone [6,7] or among postmenopausal women with higher circulating levels of estradiol and estrone [8,9]. These observations seem to suggest that P4, but not estrogen, may be the key factor for reduction of colorectal cancer risk in women.

Although intensive research has led to considerable improvement in the treatment and diagnosis at an early stage of colorectal cancer, the prognosis is still not good. Therefore, continuous searching for new therapeutic strategies is required. One approach is to identify medicinal agents capable of retarding the cell cycle and/or activating the cellular apoptotic response in the cancerous cells. Folate has been demonstrated to exert an inverse relationship between the risks of some malignancies including cancer of colon, stomach, pancreas, lung, ovary, breast and leukemia [10–12]. Epidemiological and clinical studies have shown that dietary folate supplement might decrease the risk of colorectal cancer and be involved in DNA methylation of p53 [12], suggesting a possible protective effect of folate on colorectal cancer [13,14].

Recently, we demonstrated that FA can reduce the proliferation of cultured colon cancer cell lines, such as COLO-205, HT-29 and LoVo, and inhibit the COLO-205 tumor growth *in vivo* [15]. Using

Abbreviations: AsON, antisense oligonucleotide; Con, control; FBS, fetal bovine serum; FA, folic acid; FR, folic acid receptor; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide; P4, progesterone; PR, progesterone receptor; ScON, scramble oligonucleotide; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

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COLO-205 as the cell model, our results showed that FA inhibited cell proliferation through the FRα/cSrc/ERK1/2/NFαB/p53-mediated up-regulations of p21 and p27. Interestingly, we found that this signaling pathway involved in the FA-induced anti-proliferation in COLO-205 is very similar to the signaling pathway involved in the P4-induced anti-proliferation in human umbilical venous endothelial cells [16] and rat aortic smooth muscle cells [17]. These observations led us to investigate whether P4 can affect FA's action in colorectal cancer development. Here, we demonstrated that PR activation is required for the FA-induced anti-proliferation in colorectal cancer cells.

Materials and methods

Chemicals

FA, P4, fetal bovine serum (FBS), dithiothreitol, phenylmethylsulphonyl fluoride, glycerol, Nonident P-40, sodium dodecyl sulfate (SDS), anti-ERK antibody and anti-p27 antibody were purchased from Sigma-Aldrich (St.Louis, MO). Trypsin-EDTA, penicillin-streptomycin, kanamycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), anti-phospho-serine antibody and protein A-Sepharose were purchased from Life Technologies (Darmstadt, Germany). Anti-p53, p-ERK, p21, PR, cSrc, FR and mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-cSrc antibody was purchased from Abcam (Cambridge, UK). Antibody specific for G3PDH was purchased from GeneTex (Hsinchu, Taiwan). Org 31710 (ORG) was kindly provided by Merck Sharp and Dohme Corp.

Cell culture

Five human colorectal cancer cell lines (COLO-205, HT-29, LoVo, HCT116 and DLD-1) used in this study were purchased from ATCC. These cells have performed STR-PCR profile at MB Mission Biotech. The cells were grown in a humidified incubator (37 °C, 5% CO_2). COLO-205 was growth in RPMI-1640 (GIBCO) supplemented with 10% FBS and kanamycin (100 ng/mL). HT-29, LoVo and DLD-1 were grown in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. HCT116 were grown in DMEM (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin.

MTT assay

Cell growth was estimated by a modified MTT assay. As a measurement of cell growth, the cells were seeded onto 24 well dish and grown in medium containing 10% FBS. After the cells were treated daily with FA (10 or 20 μM) for 4 days, the MTT reagent (2.5 mg/mL) was added and the optical density (570 nM) was measured by ELISA reader.

Protein extraction and western blot analysis

The cells were seeded onto 6 cm dish. After the cells had grown to subconfluence, the cells were rendered quiescent by incubation for 24 h in medium containing 0.04% FBS. The cells were released from quiescence with culture medium containing 10% FBS and treated with FA (10 or 20 μM) or PBS for the control group. The cells were washed with PBS and then lysed in lysis buffer (0.5 M Tris-HCl, pH 6.8, and 0.4% SDS). Western blot analyses were applied to determine the protein levels; the electrophoresis was performed using 8.5% or 12% SDS-polyacrylamide gel (3 h, 80 V). Separated proteins were transferred onto polyvinyl difluoride membranes (2 h, 300 mA), treated with 5% skim milk (Anchor, Auckland, NZ) to block the nonspecific IgGs, and incubated overnight at 4 °C with specific antibody. The blot was then incubated with anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories) conjugated to horseradish peroxidase for 1 h. Subsequently the polyvinyl difluoride membrane was developed with chemiluminescence reagent (Life Technologies, Darmstadt, Germany). The intensity of each band was quantified by densitometry analysis using Image Pro Plus 4.5 software.

Reverse transcription-PCR (RT-PCR)

Total cellular RNAs were extracted from the cell without any treatment. The RNA pellet was washed with 75% cold ethanol, air dried, and re-dissolved in 20 μL diethyl pyrocarbonate treated water. Two micrograms of total RNA were used in a total of 20 μL reaction volume as a template for PCR amplification. PCR was done under standard conditions in 20 μL of solution containing 10 mM Tris (pH 8.3), 40 mM KCl, 1.5 mM MgCl2, 250 μmol/L dNTP, 10 μM of each primer (sense and antisense), and one unit Taq DNA polymerase. The PCR primer sequences were as follows: FRα, forward 5′-AGCCCATAAGGATGTTTCCTA-3′, reverse 5′-TTTCATTGCACAGAACAGTG-3′, FRβ, forward 5′-CACCTCCCGCCTGTACAACTT-3′, reverse 5′-ATCTCACCAGC ATTCACATGC-3′, FRγ, forward 5′-ATGGACATGGCCTGGCAGATG-3′, reverse 5′-GGTGCTGCAGAGGGCCCCGGCCGG-3′, FRδ, forward 5′- CCTCACGACAAGCTGGGAAG-3′, reverse 5′-AGTCTTCTCACACAGGTCAGC-3′. PRA, forward 5′-ATGAGCCGGTCCGG GTGCAAGG-3′, reverse 5′-CGCCGTCGTAACTTTCGTCTTC-3′. PRB, forward 5′-CTGAGCTGAAGGCAAAGGGTCC-3′, reverse 5′-GAAGTTCGGGGCCAAACAGGCA-3′; G3PDH, forward 5′- CTCCTGTTCGACAGTCAGCC-3′, reverse 5′-TGGACTCCACGAC GTACTCA-3′. All the PCR products were analyzed using 1.5% agarose gels containing nuclear acid fluorescence to detect DNA.

Antisense oligonucleotide (AS ON)

The AS ON sequence of PR: 5′-GCTCATGAGCGGGGACAACA-3′ was designed to knock-down both PRA and PRB [18]. The Scramble oligonucleotide (Sc ON) sequence of PR: 5′-AGCACATCGAACGAGGTGGC-3′ was used for the control. Briefly, cells were seeded onto 6 cm dish and washed twice with serum-free MEM. AS or Sc ON was added in RPMI-1640 containing 5% FBS without antibiotic, and the final concentration of oligonucleotide was 10 μM. Cells were incubated with AS or Sc for 8 h, and then terminated by the addition of RPMI-1640 containing 10% FBS.

Immunoprecipitation

Immunoprecipitations were done as described previously [16]. Total proteins (300 μg) were extracted from COLO-205, and then immunoprecipitated with anti-FR (2 μg), anti-PR (2 μg) or anti-cSrc (2 μg) antibodies and protein A-Sepharose. The mixture was then gently shaken overnight at 4 °C. The complex of beads-antibody-protein was collected by pulsing vortex, washed with ice-cold PBS for 3 times, resuspended in 2X sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% dithiothreitol and 0.02% bromophenol blue), and then incubated at 98 °C for 10 min to release the proteins from the beads before electrophoresis. The bead was discarded by pulsing vortex in the centrifuge at 12,500 rpm for 10 min and the supernatant was fractionated by 8.5% SDS-PAGE. The protein–protein interaction was detected by Western blot analysis.

Statistical analysis

Values represent the means ± s.e. mean. Three to four samples were analyzed in each experiment. Comparisons were subjected to t-test and one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$

Results

Involvement of PR activation in the FA-induced growth inhibition in COLO-205

Since we have demonstrated an interaction between FA and female sex hormones in regulating endothelial cell proliferation and migration, we are interested to examine whether P4 can affect the FA-induced anti-proliferation action in colorectal cancer cells. Initially, we used COLO-205 for this study. As shown in Fig. 1A, daily treatment with FA (10 μM) or P4 (50 nM) alone for 4 days caused a significant reduction in the cell number. However, co-treatment with FA and P4 did not cause any further reduction as compared with the cell treated with FA or P4 alone, suggesting that both FA and P4 might affect the growth of COLO-205 through the same signaling pathway. To examine the involvement of PR in the FA-induced growth inhibition in COLO-205, the cell was pre-treated with Org 31710 (1 μM), a PR specific antagonist, for 1 h followed by FA (10 μM) for additional 4 days. The results show that blockade of PR abolished the FA-induced growth inhibition in COLO-205 (Fig. 1B). Since we previously demonstrated that FA caused growth inhibition in COLO-205 through up-regulations of p53, p21 and p27 induced by activating the cSrc-mediated signaling pathway, we examined the effects of PR blockade on FA-induced cSrc activation and up-regulations of p53, p21 and p27 in COLO-205. Pretreatment with Org 31710 (1 μM) abolished the FA-induced activations of cSrc and ERK1/2 (Fig. 1C) and up-regulations in p53, p21 and p27 protein (Fig. 1D) in COLO-205. These findings were confirmed by knockdown of PR using antisense oligonucleotide. Knockdown of PR prevented the FA-induced cSrc activation (Fig. 1E) and up-regulations in p53, p21 and p27 protein (Fig. 1F) in COLO-205. Taken together, these findings suggest that PR activation is involved in the FA-induced growth inhibition in COLO-205.