AMPK/p53 Axis Is Essential for α-Lipoic Acid–Regulated Metastasis in Human and Mouse Colon Cancer Cells

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Abstract: α-Lipoic acid (ALA) has an anticancer property of lung, cervix, and prostate cancer cells. However, direct evidence that ALA contributes to the development of colon cancer has not been fully elucidated. In addition, no previous studies have evaluated whether ALA may regulate malignant potential, such as adhesion, invasion, and colony formation of colon cancer cells. To address the aforementioned questions, we conducted in vitro ALA signaling studies using human (HT29) and mouse (MCA38) colon cancer cell lines. We observed that cell proliferation is reduced by ALA administration in a dose-dependent manner in human and mouse colon cancer cell lines. Specifically, 0.5 to 1 mM concentration of ALA significantly decreased cell proliferation when compared with control. Similarly, we found that ALA downregulates adhesion, invasion, and colony formation. Finally, we observed that ALA activates p53 and AMPK signaling pathways in human and mouse colon cancer cells. We found for the first time that ALA suppresses cell proliferation and malignant potential via p53 and AMPK signaling pathways in human and mouse colon cancer cells. These new and early mechanistic studies provide a causal role of ALA in colon cancer, suggesting that ALA might be a useful agent in the management or chemoprevention of colon cancer.

Key Words: α-lipoic acid, colon cancer, cell proliferation, malignant potential, p53, AMPK

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Colon cancer (also called colorectal cancer) is one of the most common malignancies in the United States, where it was ranked third in morbidity and mortality in 2013.[1] It affects approximately 1 million people each year, with a 5-year survival rate of 62%.[2,3] Even after colectomy and intensive chemotherapy, 50% of colon cancer patients have a relapse of disease, and other treatments such as radiation and chemotherapeutic procedures cause several adverse effects,[4] suggesting that developing new strategies is necessary to improve treatment.

Recent studies have shown that dietary factor and lifestyle may relate to the increasing colon cancer occurrence.[5,6] In fact, intakes of more than 20 g/d of fiber are associated with a 25% reduction of colon cancer risk and 525 mL/d of milk reduces colon cancer risk by 26% in men. By contrast, obesity increases the colon cancer incidence by 19%.[6] These reports demonstrate that colon cancer is a preventable disease through the regulation of associated risk factors. Therefore, it is important to find what substance is more effective on colon cancer risk.

α-Lipoic acid (ALA, also known as thioctic acid) is an organosulfur compound derived from octanoic acid, naturally occurring in food sources such as spinach, potatoes, broccoli, rice bran, Brewer's yeast, and so on.[7] It has been shown that ALA is a strong natural antioxidant and acts as an enzyme cofactor of the mitochondrial chain.[8] In addition, it has been suggested that ALA decreases cell proliferation and induces apoptosis in different types of tumor cells, that is, the bladder,[9,10] lung,[11,12] and breast.[13] In fact, it has been demonstrated that ALA suppresses β1 integrin expression that causes downregulation effect on cell proliferation of bladder cancer cells[9,10] and decreased tyrosine phosphorylation of EGFR, ErbB2, and Met associated with cell cycle inhibition in lung cancer cells.[11,12]

Although considerable evidence indicates that ALA has an antiproliferative effect on several cancer cells, direct evidence that ALA suppresses to the development of colon cancer, that is, cell proliferation and metastasis, has not been fully elucidated. In addition, exact signaling pathways mediating ALA's action in colon cancer development remain unknown. To address these questions, we conducted in vitro studies using human (HT29) and mouse (MCA38) colon cancer cells (1) to determine whether ALA may regulate cell proliferation and malignant potential (cell adhesion, invasion, and colony formation) and (2) to investigate whether ALA may regulate expression level of anticancer-related genes.

MATERIALS AND METHODS

Cell Culture

Human colon cancer cell lines were purchased from Korean Cell Line Bank (Seoul, South Korea). Mouse colon cancer cell lines were kindly provided by Dr. Nicholas Restifo (National Cancer Institute, National Institutes of Health). The HT29 and MCA38 cells were grown in McCoy and RPMI medium, respectively. All cells were supplemented with 10% fetal bovine serum. All cells were incubated at 37°C in an atmosphere of 5% CO_2 in the air and subcultured beyond 80% confluency.

Proliferation Assay

The cell proliferation assay was performed using the MTT proliferation kit (Sigma-Aldrich, St Louis, MO) as described by the supplier. Briefly, the cells were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were left to adhere overnight and then treated with ALA for 24 hours. Cells were washed with phosphate buffered saline (PBS) and incubated with 100 μL serum-free medium and 10 μL Vybrant MTT solution for 2 hours. Formazan crystals were dissolved overnight at 37°C with the addition of 100 μL of 10% SDS in 0.01 N HCl per well, and absorbance was measured at an optical density of 570 nm in a EMax Plus (Molecular Device, Seoul, South Korea).

Adhesion Assay

The cells were pretreated with ALA for 36 hours and plated (5×10^4 cells per well) in 10 μg/cm^2 fibronectin-coated

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(Sigma-Aldrich) wells in 96-well plates, which were then incubated at 37°C (5% CO_2) for 60 minutes. Adherent cells were fixed with 3% paraformaldehyde for 10 minutes, washed with 2% methanol for 10 minutes and stained with 0.5% crystal violet in 20% methanol for 10 minutes. The stain was eluted and absorbance was measured at 540 nm.

Invasion Assay

For an in vitro model system for metastasis, we performed a Matrigel invasion assay by using a Matrigel invasion chamber from BD BioCoat Cellware (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

Clonogenic Assay

The cells were grown in the medium to 60% confluency and treated with ALA for 24 hours. After 24 hours, 500 cells were reseeded into a 100-mm culture dish and incubated for 12 days. Fresh medium was changed every 3 days. At day 12, the medium was removed, added to 2 mL of Clonogenic Reagent (50% ethanol + 0.25% 1,9-dimethyl-methylene blue), and was left at room temperature for 45 minutes. After 45 minutes, the cells were washed with PBS, and the blue colonies were counted.

Western Blot

After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc, Keene, NH). The membranes were blocked for 1 hour in tris-base buffered saline (TBS) containing 5% nonfat dry milk and 0.1% Tween 20. Incubation with primary antibodies was performed in TBS containing 5% nonfat dry milk overnight and then incubated with horseradish peroxidase secondary antibodies for 2 hours. After incubation with antibodies, membranes were washed with TBS containing 0.1% Tween 20. Enhanced chemiluminescence was used for detection. Measurement of signal intensity on nitrocellulose membranes after Western blotting with various antibodies was performed using Image J processing and analysis software (http://rsbweb.nih.gov/ij/).

Statistical Analysis

All data were analyzed using Student *t* test and/or 1-way analysis of variance followed by post hoc tests (Bonferroni correction for multiple comparisons). All analyses were performed using SPSS version 11.5 (SPSS, Chicago, IL).

RESULT

Regulation of Cell Proliferation by ALA in Human and Mouse Colon Cancer Cell Lines

To investigate the effect of ALA on the cell viability of human HT29 and mouse MCA38 colon cell lines, we treated cells with different concentrations of ALA (0.5, 1, and 5 mM) and performed MTT assay. We observed that cell proliferation was downregulated

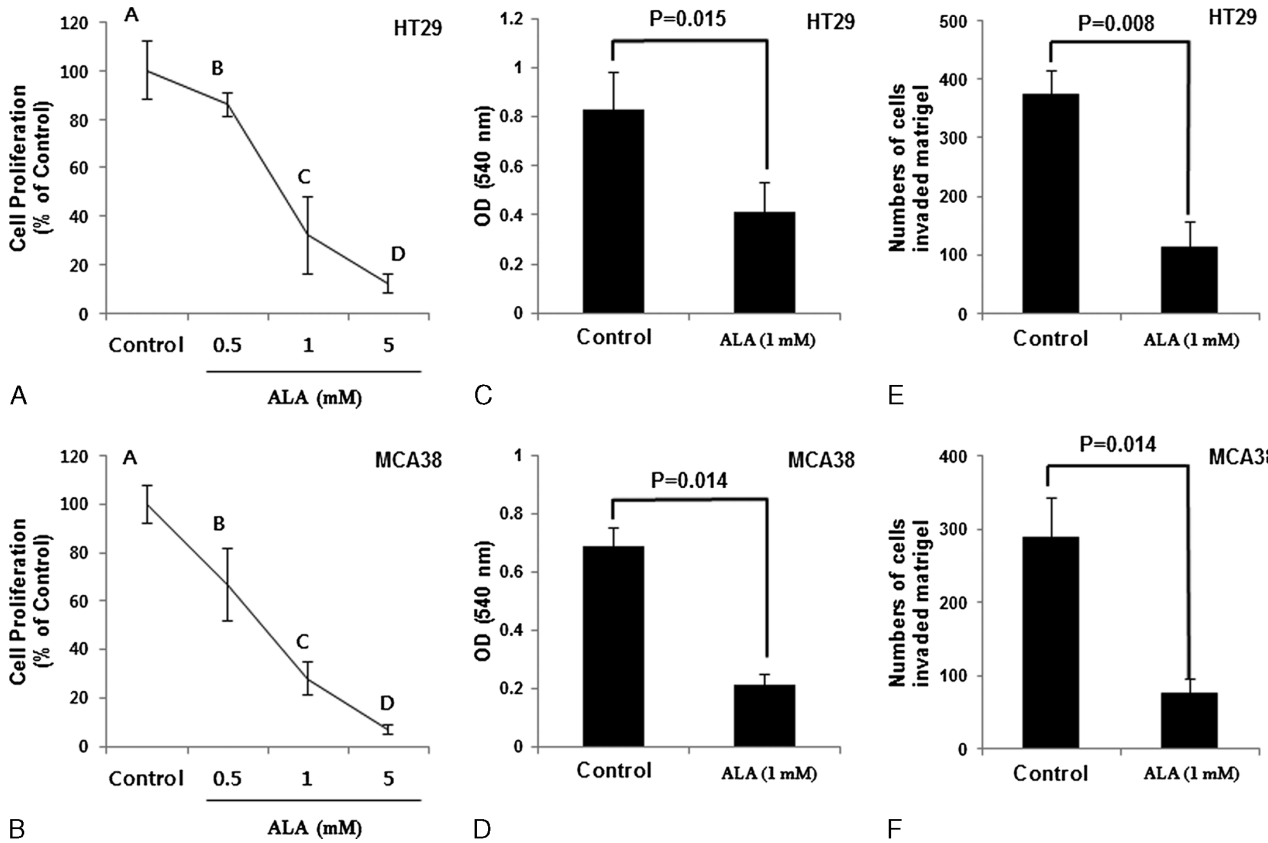

FIGURE 1. Regulation of cell proliferation, adhesion, and migration by ALA in human (HT 29) and mouse (MCA 38) colon cancer cell lines. A and B, The cells were treated with ALA at indicated concentrations for 36 hours, and MTT assay was then performed as described in Methods; (C–F) The cells were treated with ALA (1 mM), and (C–D) cell adhesion and (E–F) invasion assays were then performed as described in Methods. All data were analyzed using Student *t* test and/or 1-way analysis of variance followed by post hoc test for multiple comparisons. Values are presented as mean (SD) (n = 3). Means with different letters are significantly different, $P < 0.05$, whereas means with similar letters are not different from each other.

by ALA in a dose-dependent manner (Fig. 1, A and B). Specifically, treatment with 1 mM of ALA decreased by approximately 40% to 50% of cell proliferation when compared with control and most cells detached when administered with high concentration of ALA (5 mM). Based on these results, we chose 1 mM of ALA as a representative concentration and performed malignant potential assays (see below).

Regulation of Malignant Potential by ALA in Human and Mouse Colon Cancer Cell Lines

In addition, to examine the effect of ALA on cell proliferation, we performed cell adhesion assay. We observed that ALA-treated cells had effectively decreased cell adhesion activity when compared with control (Fig. 1, C and D). For an in vitro model system for metastasis, we performed a Matrigel invasion assay by using a Matrigel invasion chamber. As shown in Figures 1E and 1F, ALA showed a significant inhibitory effect on cell invasion activity of both human and mouse colon cancer cell lines. Next, we also examined the growth inhibitory effects of ALA on human and mouse colon cancer cells by a long-term colony formation assay, showing that ALA significantly reduced colony number (Fig. 2A). We did not observe any major differences in magnitude of all malignant potential activities in response to ALA between human and mouse colon cancer cell lines.

Regulation of p53 and AMPK Signaling by ALA in Human and Mouse Colon Cancer Cell Lines

To understand the potential molecular signaling events underlying the antiproliferative effects on ALA, we examined the expression levels of AMPK and p53 by Western blot analysis. As shown in Figures 2B and 2C, ALA treatment caused sharp activation in p53 and AMPK expression. Specifically, the ratio of p-p53/p53 and p-AMPK/AMPK in colon cell lines was increased almost 3 times higher than control. These results suggest that the induction of apoptosis in human and mouse colon cell lines treated with ALA was possibly due to the alteration in AMPK and p53 signaling pathway.

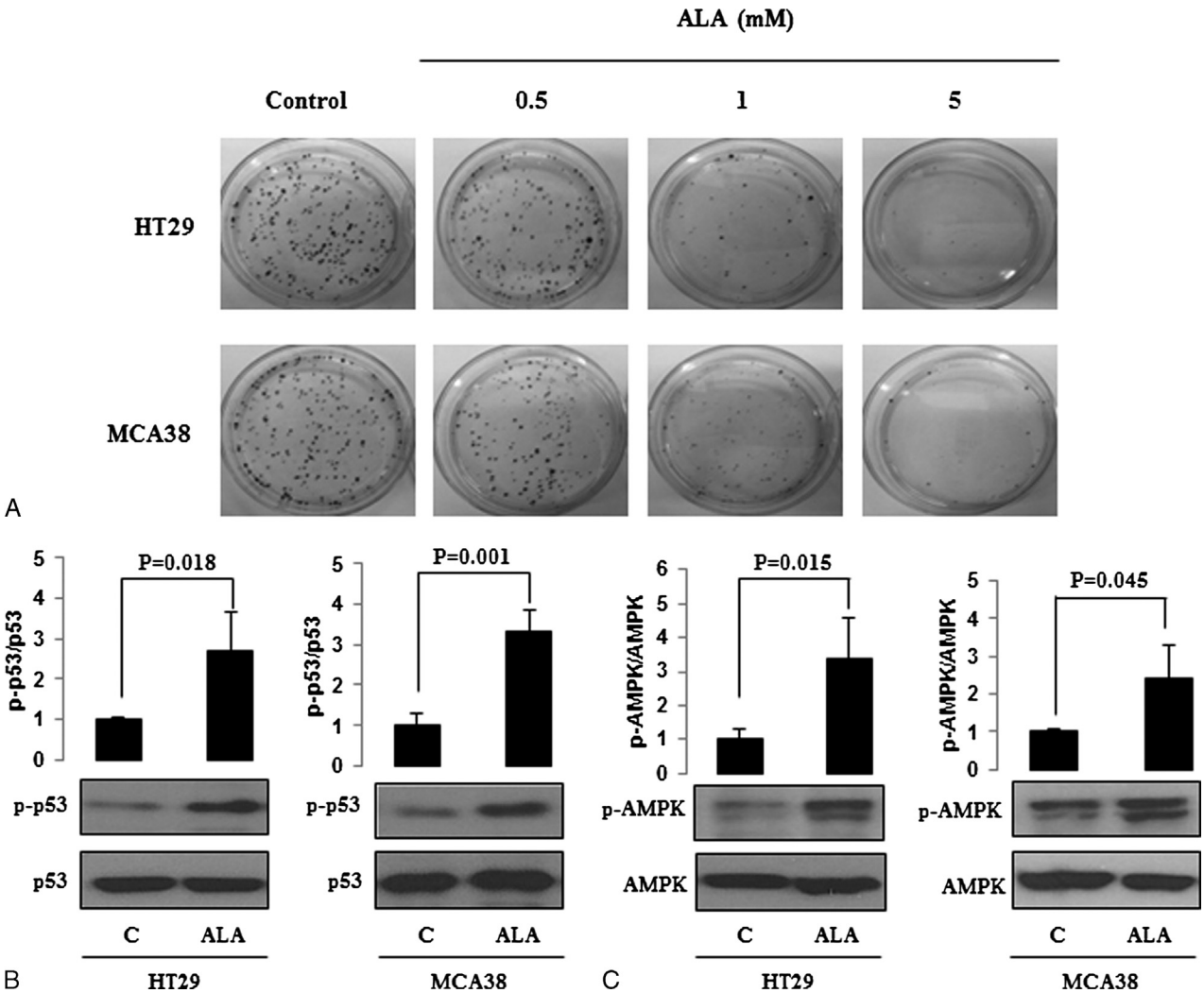

FIGURE 2. Regulation of colony formation and intracellular signaling pathways by ALA in human (HT 29) and mouse (MCA 38) colon cancer cell lines. A, The cells were treated with ALA at indicated concentration for 12 days, and colony formation assay was then performed as described in Methods. B and C, The cells were treated with ALA (1 mM) for 30 minutes, and cell lysates were examined by Western blotting with primary p53 (mouse), p-p53 (mouse), AMPK (goat), and p-AMPK (mouse) antibodies as described in Methods. The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-goat antibodies. All density values for each protein band of interest are expressed as a fold increase. All data were analyzed using Student *t* test. Values are presented as mean (SD) (n = 3).

DISCUSSION

α-Lipoic acid is organic sulfur compound found in many common vegetable.[7] It is derived from octanoic acid through many fatty acid biosynthesis.[14] α-Lipoic acid is a nutritional coenzyme involved in energy metabolism.[14] Specially, ALA has been shown to improve mitochondrial metabolism as part of several multienzyme complex.[15] In addition, ALA acts as an antioxidant agent because it can extend the activity of vitamins.[16] Furthermore, recent studies have suggested that ALA has a beneficial effect on cancers such as bladder cancer, lung cancer, and breast cancer.[9–13] By contrast, whether ALA may have an anticancer effect not only by altering cell proliferation and malignant potential but also through anticancer-related signaling pathways on human and mouse colon cancer cells has not yet been elucidated.

To avoid being eliminated from circulation, cancer cells move to establish new metastatic colonies.[17] Hence, adhesive interactions are important to the process of metastatic tumor dissemination because cell adhesion and colonization occurs during cancer metastasis.[17] We found for that first time that ALA reduces cell adhesion activity and decreases the number of colonies formed in human and mouse colon cancer cell lines. These data demonstrated that ALA is capable of preventing the metastasis of colon cancer by controlling cell adhesion and colony formation. Among the discrete stages of metastasis, the invasion of primary cancer cells has received intensive investigation, and the microtubule dynamics has emerged as a promising anti-invasion target.[18,19] Based on these previous reports, we performed migration assay using Matrigel invasion chamber as an in vitro model system of metastasis. We observed that ALA decreases cell invasion activity in both human and mouse colon cancer cells when compared with control, suggesting that ALA may affect colon cancer metastasis.

AMPK plays a key role in regulation of inflammation, metabolism, and tumor progression.[20] Because colon cancer has a strong inflammatory and metabolic component, AMPK activation may be useful in tumor management.[20] In addition, it is well known that p53 is a tumor suppressor gene in response to DNA damage, oncogene activation, and hypoxia by inducing cell cycle arrest.[20] Importantly, because AMPK can directly phosphorylate p53, regulation of AMPK/p53 signaling may be a key axis in terms of generating the cancer development.[20] We observed that ALA increases phosphorylation level of p53 and AMPK in human and mouse colon cancer cell lines. These results suggest that ALA-induced p53 and AMPK activation is an important signaling mechanism underlying suppressed growth in both human and mouse colon cancer cells that is associated with metastasis.

In summary, we report for the first time that ALA regulates the malignant potential (proliferation, adhesion, invasion, and colony formation) of human and mouse colon cancer cell lines. In addition, we demonstrated that the anticancer effect of ALA is dependent on p53/AMPK signaling pathways. These results suggest that ALA-reduced metastasis may interact with p53/AMPK axis in colon carcinogenesis, and this needs to be studied further by future investigations. Because we actually did not look at all signaling pathways, much more work needs to be done in the future. Despite this limitation, this early and novel mechanistic study provides evidence for a causal role of ALA in colon cancer and indicates that if the data are confirmed and extended by future studies, ALA could potentially prove to be a useful agent in the management of colon cancer. Finally, in vivo studies in rodents, and hopefully later in humans, could further elucidate the role of ALA's potential use as a therapeutic agent for colon cancer.

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