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Effects of Steady Low-Intensity Exercise on High-Fat Diet Stimulated Breast Cancer Progression Via the Alteration of Macrophage Polarization

Min Kyoon Kim, MD, PhD[1*], Yesl Kim, MS[1*], SeungHwa Park, MS[1], Eunju Kim, PhD[2], Yerin Kim, MS[2], Yuri Kim, PhD[2], and Jung-Hyun Kim, PhD[1]

Abstract

Physical inactivity and high-fat diet, especially high saturated fat containing diet are established risk factors for breast cancer that are amenable to intervention. High-fat diet has been shown to induce tumor growth and metastasis by alteration of inflammation but steady exercise has anti-tumorigenic effects. However, the mechanisms underlying the effects of physical activity on high-fat diet stimulated breast cancer initiation and progression are currently unclear. In this study, we examined how the intensity of physical activity influences high fat diet-stimulated breast cancer latency and progression outcomes, and the possible mechanisms behind these effects. Five-week-old female Balb/c mice were fed either a control diet or a high-fat diet for 8 weeks, and then 4T1 mouse mammary tumor cells were inoculated into the mammary fat pads. Exercise training occurred before tumor cell injection, and tumor latency and tumor volume were measured. Mice with a high-fat diet and low-intensity exercise (HFLE) had a longer tumor latency period, slower tumor growth, and smaller tumor volume in the final tumor assessment compared with the control, high-fat diet control (HFDC), and high-fat diet with moderate-intensity exercise (HFME) groups. Steady low- and moderate-intensity exercise had no effect on cell proliferation but induced apoptosis by activating caspase-3 through the alteration of Bcl-2, Bcl-xL, and Bax expression. Furthermore, steady exercise reduced M2 macrophage polarization in breast tumor tissue, which has been linked to tumor growth. The myokine, myostatin, reduced M2 macrophage polarization through the inhibition of the JAK-STAT signaling pathway. These results suggest that steady low-intensity exercise could delay breast cancer initiation and growth and reduce tumor volume through the induction of tumor cell apoptosis and the suppression of M2 macrophage polarization.

Keywords

breast cancer, high-fat diet, low-intensity exercise, latency, macrophage polarization, myostatin

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among women.[1,2] Breast cancer risk has been associated with excessive adiposity, physical inactivity, and poor diet.[3-5] High fat diet has been shown to induce the incidence, growth, and metastasis of breast cancer by inducing low-grade chronic systemic inflammation in animal models.[5-7] Thus moderate consumption of fat through a healthy diet and exercise are recommended to reduce the risk of breast cancer.[3,4]

Exercise training in women with early-stage breast cancer has been reported to enhance aerobic capacity, muscular strength, and physical function, improve the quality of life, and reduce anxiety and depression.[8] In addition to this, observational research suggests that physical activity and exercise training may reduce the risk of breast cancer. Several biological pathways may protect against cancer development or progression, including systemic low-grade inflammation

[1]Chung-Ang University, Seoul, Korea
[2]Ewha Womans University, Seoul, Korea

*Contributed equally to this work.

Corresponding Author:
Jung-Hyun Kim, Department of Physical Education, Chung-Ang University, 84 Heuksuk-Ro, Dongjak-Gu, Seoul 06974, Korea.
Email: jjhkim@cau.ac.kr

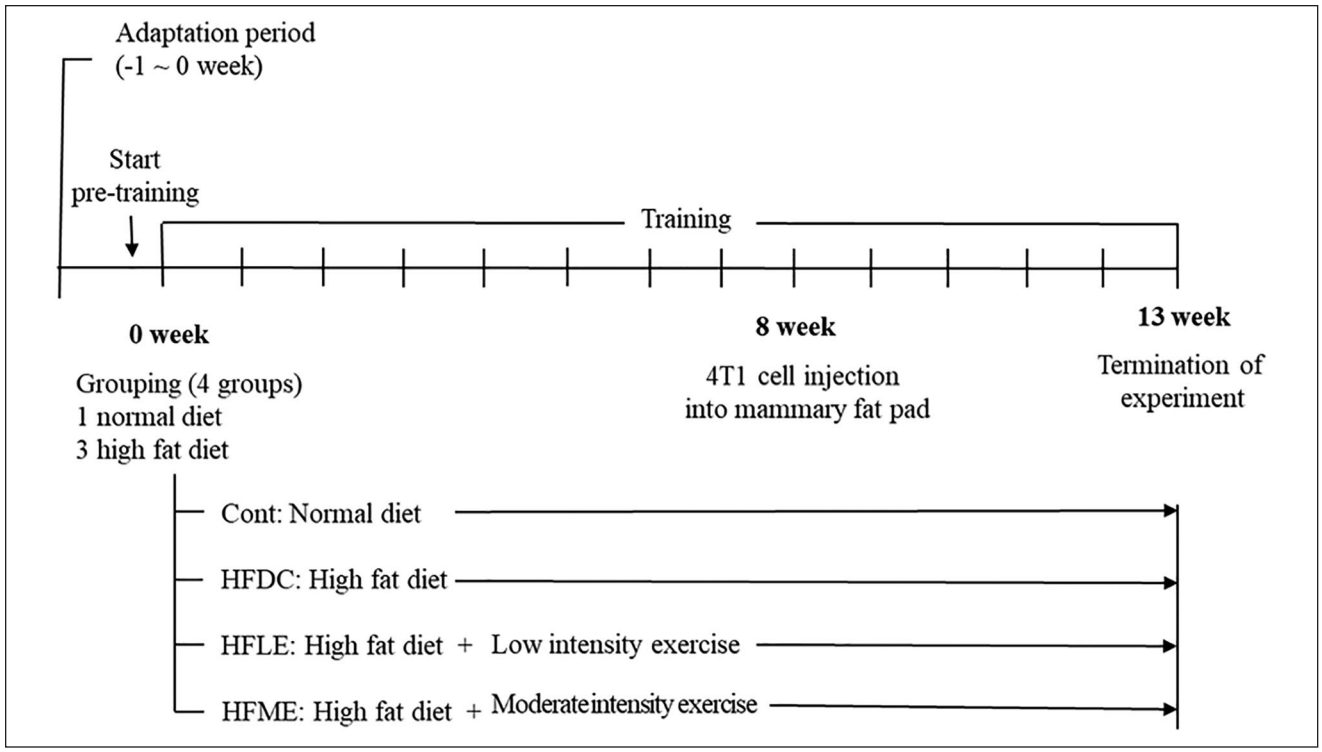

Figure 1. Experimental study design. After 1 week of adaptation, 8-week-old Balb/c mice were fed either a control diet or a high-fat diet for 8 weeks, and then 4T1 mouse mammary tumor cells (1×10^4) were transplanted into their mammary fat pads. The low- and moderate-intensity exercise groups were pre-trained for 5 days. The HFLE and HFME groups were trained 5 days a week until the experiments ended.

and alterations in sex hormones, metabolic hormones, and DNA repair capacity.[9-11] In the case of physical activity, several studies have demonstrated that exercise induces the release of cytokines from working muscles.[12] The production of these muscle-derived cytokines, which are known as myokines, is evidence of the fact that muscle tissue has an inherent ability to function as a secretory organ.[13] However, the anti-cancerous effects of physical activity are not yet entirely understood, particularly the mechanisms underlying the effects on breast cancer initiation and progression.

The tumor microenvironment provides suitable conditions for all stages of cancer, including initiation, progression, epithelial-mesenchymal transition, and metastasis.[14] Macrophages are immune cells that play a powerful role in tumor microenvironments.[15,16] Cytotoxicity of macrophages reduced tumor during the early immune response, however, macrophages in most solid tumors potentiated tumor progression. Macrophages in tumor tissue, as called tumor-associated macrophages (TAMs), were inversely correlated with clinical outcome and survival in various cancers through stimulation of tumor progression and angiogenesis.[17] Especially, the M2 form of TAMs secretes anti-inflammatory cytokines and growth factors that are crucial for pro-tumorigenic processes.[15,17-20] Since a tumor mass includes many M2 form of macrophages, they can be a specific target for cancer treatment.[15,21] In particular, inhibiting the infiltration and M2 polarization of TAMs represents a potential anticancer therapy.

A member of the transforming growth factor β (TGF-β) superfamily, myostatin is a myokine that regulates muscle development.[22] Recent studies have shown that myostatin is highly expressed in low-grade breast adenocarcinoma, but its expression levels fall in higher-grade malignancies.[23] In addition, greater expression of myostatin is associated with a higher survival rate for breast cancer patients and plasma myostatin levels are positively associated with amounts of muscle mass.[23,24] However, the mechanisms underpinning the preventive effect of myostatin on breast cancer have not yet been elucidated. Therefore, in this study, we investigate the role of long-term exercise training at different intensities in the development of breast cancer and the possible mechanisms involved.

Materials and Methods

Experimental Animals and Diets

The experimental study design is presented in Figure 1. Five-week-old female Balb/c mice were obtained from Koatech

Inc. (Gyeonggi-do, Korea). The mice were immediately segregated by weight and randomly placed into 4 groups (n = 9 per group): (i) a normal control group given AIN93G (Table 1), (ii) a high-fat diet control group (HFDC), (iii) a high-fat diet with low-intensity exercise (HFLE) group, and (iv) a high-fat diet with moderate-intensity exercise (HFME) group. Exercise training was initiated after a weeklong adjustment period on either the control (Unifaith Inc., Seoul, Korea) or high-fat diets (Research Diets, USA). All mice were pre-trained on treadmills for 1 week and then underwent training before and after tumor cell inoculation (Figure 1). The exercise groups of animals were trained at either a low intensity (10 m/min) or a moderate intensity (15 m/min on a slope of 2.5°) for 5 days per week. The mice were housed in clean cages with controlled temperature and humidity (22 ± 3°C and 55 ± 5%) and a 12/12-hour light-dark cycle. The procedures used were in accordance with institutional guidelines and approved by the Chung-Ang University Institutional Review Board (ID No. 2016-00099, Seoul, Korea).

Tumor Cells and U937 Cell Cultures

The murine mammary cancer cell line 4T1 was obtained from Prof. Seungmin Lee, and U937 cells were obtained from the ATCC (Manassas, VA, USA). 4T1 and U937 cells were maintained in culture using either Roswell Park Memorial Institute 1640 (RPMI 1640; WelGENE, Korea) containing 10% fetal bovine serum (FBS; Gibco, USA), 1% non-essential amino acids (WelGENE, Korea), and 1% penicillin/streptomycin (P/S; WelGENE, Korea) or high-glucose (25 mM) RPMI medium containing 10% FBS with 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. U937 cells were differentiated into macrophages using 10 ng/mL of phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, MO, USA) for 48 hours, followed by treatment with 10, 50, or 100 ng/mL of myostatin 2 hours before 30 ng/mL of IL-4 (Peprotech, Rocky Hill, NJ, USA) for 48 hours to induce M2 macrophage polarization.

Tumor Cell Transplantation

For the transplantation of tumor cells, mice that had been fed their assigned diets for 8 weeks were hypodermically inoculated in the mammary fat pad with 4T1 cells (1×10^4) suspended in RPMI 1640 media that had been grown in culture to 50% confluency. Tumor volume (v) was measured twice a week and calculated as follows:

$$\text{Tumor volume} = \text{longest diameter} \times \text{shortest diameter} \times \text{height} \times 0.5236$$

Diameter measurements were reduced by 1 mm to account for the skin. Latency was calculated as the time for the tumor volume to reach 8 mm^3. After 5 weeks of tumor cell inoculation, the mice were sacrificed via inhalation anesthesia using isoflurane (Piramal Critical Care, Bethlehem, PA, USA). The tumor tissue, liver, and spleen tissues were immediately dissected, washed with ice-cold PBS, and weighed.

Cell Viability Assay

Cell viability was analyzed by the Trypan blue exclusion assay. Cells were cultured in 12-well plates and treated with PMA for 48 hours, followed by treatments with 10, 50, or 100 ng/mL of myostatin 2 hours before 30 ng/mL of IL-4. After cells were trypsinized and centrifuged at 500 × g for 5 minutes, pellets were resuspended in culture media. Trypan blue solution (Invitrogen) was added into the cell suspension and a final concentration was 0.2%. Dead cells were stained blue, whereas the viable cells remained unstained. The proportion of viable cells was calculated by dividing the number of live cells by the number of total cells.

Western Blotting Analysis

The tumor tissue and U937 cells were separately homogenized with ice-cold RIPA buffer (Sigma, Seoul, Korea) or scraped into cold PRO-PREP protein extract solution (Intron Biotechnology, Seoul, Korea). After centrifugation (13 000 × g, 15 minutes, and 4°C), the supernatant was collected and stored at −80°C. The total protein concentration of the supernatant was quantified using a colorimetric protein assay kit (BIO-RAD, CA, USA). Denatured proteins were separated on SDS-PAGE gel. After electrophoresis, the proteins were transferred to polyvinylidenedifluoride membranes, which were blocked in 5% skim milk or 5% BSA in Tris-buffered saline with Tween-20 (TBST) buffer for 1 hour. The membranes were incubated with PCNA, Bcl-2, Bcl-xL, Bax (Santa Cruz Biotechnology, CA, USA), caspase-3 (Cell Signaling, MA, USA), CD163 (Abcam, Cambridge, England), phospho-Janus kinase 1 (p-JAK1), total-JAK1, phospho-signal transducer and the activator of transcription 6 (p-STAT6), and total-STAT6 (Cell Signaling, MA, USA) antibodies overnight at 4°C. After the membranes were washed with TBST buffer, the membranes were incubated for 1 hour with a secondary antibody (Santa Cruz Biotechnology, CA, USA) at room temperature. Immuno-detection bands were reacted with an enhanced chemiluminescence (ECL) reagent (Energenesis Biomedical Co., Ltd, Taipei City, Taiwan). The levels of β-actin and α-tubulin (Sigma-Aldrich, MO, USA) were used as the reference control.

RNA Isolation and Real-Time Polymerase Chain Reaction analysis

Total RNA was isolated from U937 cells using TRIzol reagent (Invitrogen, CA, USA), and cDNA was synthesized via reverse transcription using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA).

Traditional polymerase chain reaction (PCR) was performed according to the manufacturer's instructions with Taq polymerase (TAKARA, Tokyo, Japan) and an SYBR Green kit. The resulting PCR products were separated in 2% agarose gel containing GoodView Nucleic Acid Stain (SBS Genetech Co. Ltd, Beijing, China) and visualized under an ultra-violet lamp. The primers used for PCR analysis were human CD163, 5′-CCAAAATCCAGGCAACA AAC-′3 (forward) and 5′-GCTTCACTTCAACACGTCCA -′3 (reverse); human Arg1, 5′-GGTGATGGAAGAAACAC TCA-′3 (forward) and 5′-GTAGCTGGTGTGAAAGATGG -′3 (reverse); and human GAPDH, 5′-AGAAGGCTGGGGC TCATTTG-′3 (forward) and 5′-AGGGGCCATCCACAG TCTTC-′3 (reverse). GAPDH, a housekeeping gene, was detected as a loading control.

Immunohistochemistry

Tumor tissues were fixed in 10% formaldehyde and embedded in paraffin. Serial histological sections (5-μm thick) were deparaffinized and hydrated in descending dilutions of ethanol. Antigen retrieval was achieved by autoclaving at 121°C for 20 minutes in citrate buffer (pH 6.0) and endogenous peroxidase was blocked by 3% H_2O_2. Sections were then covered with 1% BSA at RT for 30 minutes then incubated with CCR7 or CD163 antibodies Novus Biologicals, CO, USA) at 4°C overnight. Horseradish peroxidase labelled anti-goat/rabbit (Dako, CA, USA) antibodies were applied to the slides at RT for 1 hour, followed by the Vectastain ABC Kit (Vector Laboratories, Burlingame, USA) at RT for 30 minutes according to the manufacturer's instructions. The reaction products were detected by 3,3′-diaminobenzidine (Dako), and then counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries, Osaka, Japan). After being dehydrated by ascending dilutions of ethanol, the slides were immersed in xylene, and mounted.

Statistical Analysis

Experiments were repeated at least three times, and results were presented as the mean ± standard deviation (SD). All data were statistically analyzed using 1-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. In all analyses, results were considered significant if the *P* value was less than .05. All statistical tests were performed using the statistical analysis software SPSS 25.0 (Chicago, IL, USA).

Results

Effect of Aerobic Exercise on Breast Tumor Latency and Growth

We investigated whether chronic low- or moderate-intensity aerobic exercise had any effect on the initiation of high-fat diet stimulated breast tumor formation and tumor growth in an animal model. We first assessed whether steady exercise modulated breast tumor initiation and growth. The latency period after grafting was determined to be the number of days it took for the tumors to reach a size of 8 mm^3. The animals in the control and HFDC groups took about 7.3 and 7.2 days on average, respectively, to reach the target tumor size (Figure 2A). On the other hand, the latency period for animals subject to low-intensity exercise was 12.3 days after tumor cell inoculation. To assess the effect of steady exercise on breast tumor growth, mean tumor volume was calculated and compared with the control group (Figure 2B). As early as 16 days after inoculation, the tumor growth of the HFLE and HFME groups was slower, with a conspicuously smaller tumor volume, compared with the control and HFDC groups. After 36 days, HFLE but not HFME showed a significant decrease in tumor volume compared with the control and HFDC specimens. Overall, steady low-intensity

Table 1. Ingredients of the Experimental Diets (g/Kg Diet).

Ingredient	Control diet[a]	High-fat diet
Corn starch	397.5	—
Dextrin	132	116.4
Sucrose	100	77.4
Casein, lactic	200	200
Cellulose	50	50
Soybean oil	70	25
Lard	—	245
Mineral mix[b]	35	10
Dicalcium phosphate	—	13
Calcium carbonate	—	5.5
Potassium citrate	—	16.5
Vitamin mix[c]	10	10
Choline bitartrate	2.5	2
L-cystine	3	3
Leucrose	—	—
H_2O	—	16.5
Total amount (kg)	1	1
Energy (kcal/kg)	4	5.243

[a]All amounts were based on an AIN-93G diet.
[b]Mineral mix: calcium carbonate (35.7%), potassium phosphate monobasic (25.0%), potassium citrate monohydrate (2.8%), sodium chloride (7.4%), potassium sulfate (4.66%), magnesium oxide (2.43%), ferric citrate (0.606%), zinc carbonate (0.165%), manganous carbonate (0.063%), cupric carbonate (0.031%), potassium iodate (0.001%), sodium selenite (0.001%), ammonium paramolybdate (0.001%), sodium metasilicate (0.145%), chromium potassium sulfate (0.028%), lithium chloride (0.002%), boric acid (0.008%), sodium fluoride (0.006%), nickel carbonate hydroxide tetrahydrate (0.003%), ammonium vanadate (0.001%), and sucrose (20.95%).
[c]Vitamin mix: nicotinic acid (0.3%), calcium pantothenate (0.16%), pyridoxine-HCl (0.06%), riboflavin (0.06%), folic acid (0.02%), D-biotin (0.002%), Vit B12 in 0.1% mannitol (0.25%), DL-α-tocopherol acetate, 500 IU/g (1.50%), retinol palmitate, 500 000 IU/g (0.08%), Vit D3 (50 000 IU/g) (0.02%), Vit K (0.007%), and sucrose (97.47%).

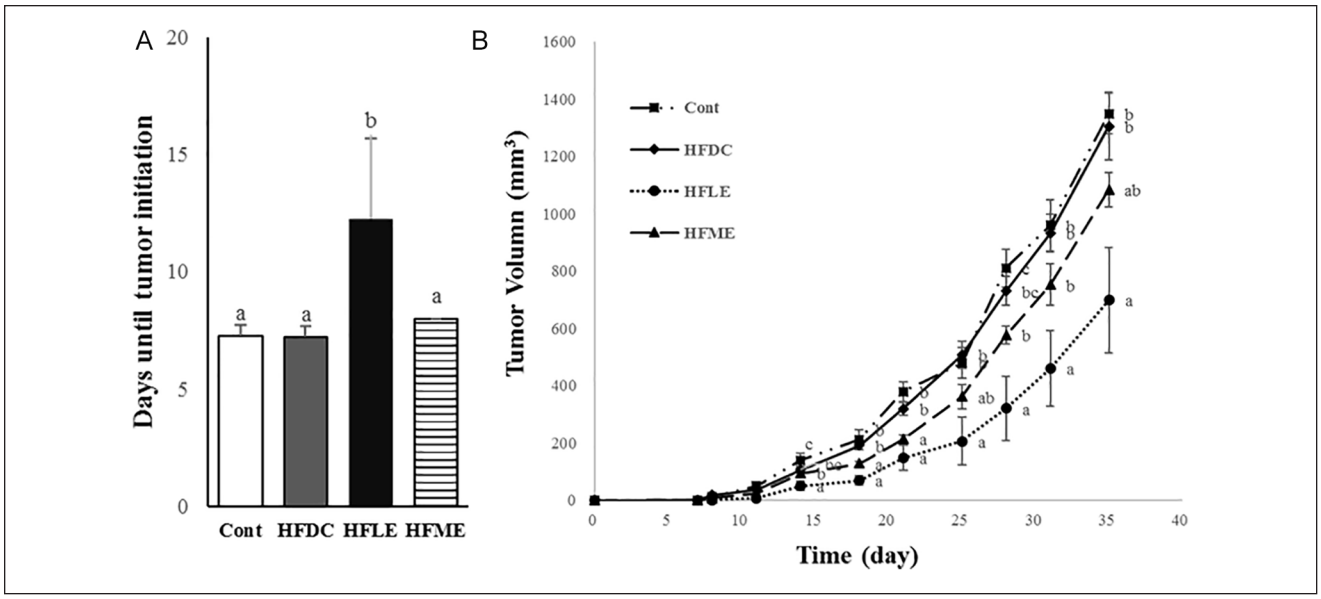

Figure 2. Effects of low- and moderate-intensity exercise on breast tumor latency and growth. Mice were fed either the control diet or high-fat diet and exercised at low- or moderate-intensity for 8 weeks; tumor cells were then transplanted into the mice. (A) After inoculation with 1×10^4 4T1 cells, latency was calculated as the time for the injected tumor cells to reach a size of 8 mm^3. (B) Tumor volume was measured twice a week and calculated until they reached 1000~1500 mm^3. Values are presented as the mean ± SEM. Values not sharing the same superscript were significantly different ($P < .05$).

Table 2. Effects of Low- and High-Intensity Exercise on Organ and Breast Tumor Weight.

	Control	HFDC	HFLE	HFME
Body weight				
Initial BW (g)	17.52 ± 0.42	17.72 ± 0.62	17.82 ± 0.30	17.87 ± 0.83
BW before TI (g)	19.82 ± 0.35	20.95 ± 1.42	20.52 ± 1.59	21.52 ± 0.92
Final BW	19.40 ± 0.43	19.85 ± 1.29	20.55 ± 1.38	20.22 ± 0.61
Liver (g)	1.04 ± 0.07[a]	0.95 ± 0.06[a,b]	0.86 ± 0.04[b]	0.89 ± 0.01[b]
Spleen (g)	0.71 ± 0.16	0.57 ± 0.08	0.44 ± 0.16	0.59 ± 0.12

Abbreviations: TI, tumor inoculation; BW, body weight. Values not sharing the same superscript were significantly different ($P < .05$).

exercise appears to be effective for reducing breast tumor latency and growth.

Effects of Low- and Moderate-Intensity of Exercise on Body Weight and Organ Weight

Body weight and organ weight were measured to identify differences among experimental groups. As shown in Table 2, there were no significant body weight differences among the groups. Spleen weight was measured as an indirect indicator of inflammation; the spleen is an immunological tissue that increases in size during various inflammatory challenges including infection and cancer, initiating an immune response directed towards specific tissues that need repair.[25] Spleen and liver weights were lower in the HFLE groups.

Regulation of Breast Tumor Growth Using Steady Exercise

We analyzed whether the inhibitory effect of steady low-intensity exercise on breast tumors modulated tumor cell proliferation and/or apoptosis. Thus, the expression of protein markers related to proliferation and apoptosis was tested in tumor tissue (Figure 3A). There were no significant differences of the proliferation marker, PCNA, expression among the groups (Figure 3B). However, the expression of the anti-apoptotic proteins Bcl-2 (Figure 3C) and Bcl-xL (Figure 3D) was significantly lower in the exercise groups than in the control group. In addition, the expression of the pro-apoptotic protein marker Bax was significantly higher in the low-intensity exercise group than in the HFDC and HFME groups (Figure 3E). As a consequence of modulated

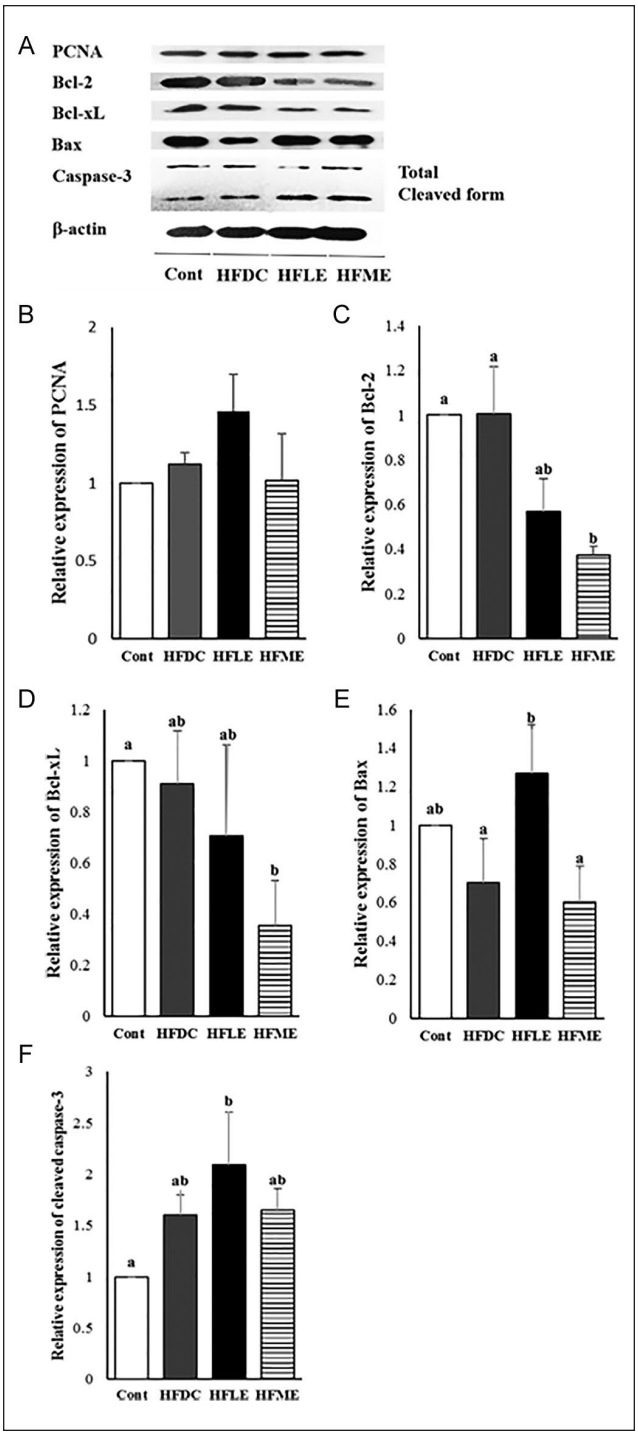

Figure 3. Effects of low- and moderate-intensity exercise on tumor cell proliferation and apoptosis (A-F). Tumor tissues from the 4 groups were used to measure PCNA (B), Bcl-2 (C), Bcl-xL (D), Bax (E), and caspase-3 (F) expression via Western blot.

apoptosis regulating protein expression, steady low-intensity exercise significantly increased the active form of caspase-3 compared with the control group (Figure 3F).

Myostatin Inhibition of M2 Macrophage Polarization by Regulating the JAK1/STAT6 Pathway

It was observed that TAMs modulated breast tumor growth and that M2 macrophages increased tumor growth and metastasis through the induction of chronic inflammation. Based on these results, we investigated whether variation in the intensity of steady exercise modulated TAM polarization in tumor tissues (Figure 4A). Low- and moderate-intensity exercise significantly decreased the number of M2 macrophages (CD163+) but had no effect on M1 macrophages (CCR7+). Since exercise modulates myokine secretion from the muscle, we also investigated whether myostatin, a myokine which modulates breast tumor growth and mesenchymal stem cell differentiation, was involved in inhibiting M2 macrophage polarization using U937 cells. Following PMA treatment, U937 cells exhibited certain characteristics of polarized macrophages and IL-4 increased the presence of M2 polarization markers. Myostatin had no cytotoxic effects on cell viability compared to PMA treatment control (Figure 4B). The mRNA levels of M2-associated markers, including CD163 and Arg1, were significantly lower following myostatin treatment when compared with the IL-4 group (Figure 4C). In particular, 100 ng/mL of myostatin significantly downregulated CD163 and Arg1 by 45.4% and 65.9%, respectively. Western blotting analysis confirmed that myostatin reduced M2 macrophage polarization (Figure 4D). CD163 expression was 70.5% lower in the M100 group. In addition, the phosphorylation of both JAK1 and STAT6 signaling was downregulated following myostatin treatment by 65.3% and 71.5%, respectively, a significant difference compared with the IL-4 group.

Discussion

In this study, we show that the intensity of physical exercise may influence breast cancer progression. Long-term, low-intensity exercise before tumor inoculation resulted in a longer tumor latency period and a smaller final tumor volume compared with the other exercise interventions. In addition, myokines, which are secreted from working muscle, modulated the tumor microenvironment by affecting TAM polarization.

Steady low-intensity exercise reduced tumor growth by inducing apoptosis. A meta-analysis has reported that physical activity pre- and post-diagnosis affects patient survival through different mechanisms.[26] In that analysis, pre-diagnosis physical activity appeared to reduce breast cancer mortality only in patients with a BMI of less than 25 kg/m^2, but post-diagnosis activity only benefited obese women. In addition, post-diagnosis physical activity had no beneficial effects on outcomes among women with

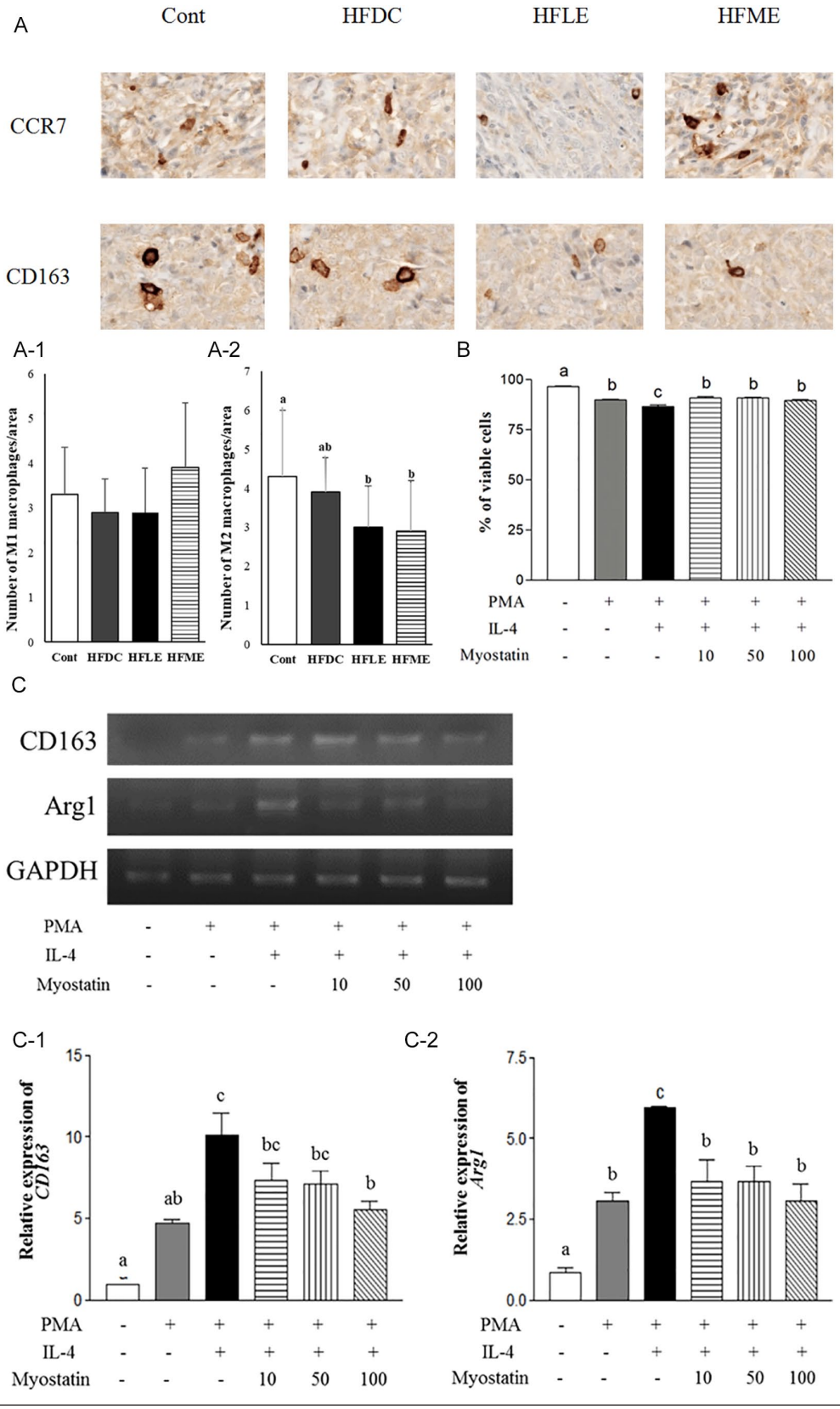

Figure 4. *(continued)*

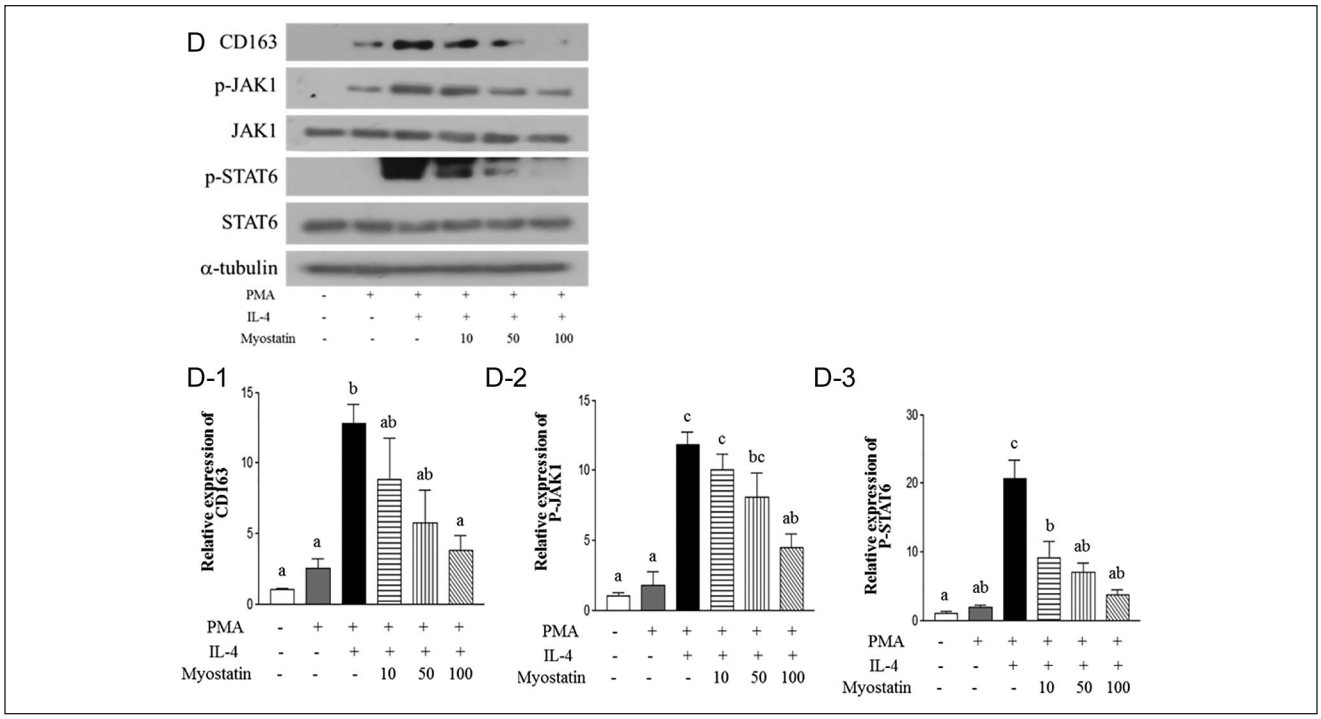

Figure 4. Effects of exercise and myostatin on tumor associated macrophage polarization on tumor tissues (A) and U937 cells (B-D). (A) Tumor tissues from the 4 groups were used to measured CCR7 and CD163. Original magnification: 400×. (B) Cell viability, (C) mRNA expression of CD163 and Arg1, and (D) levels of CD163 and phosphorylation of JAK1/STAT6 were measured in the macrophages differentiated from U937 cells using trypan blue exclusion assay and Western blotting assays. Representative blots (left panel) and the quantification of the band (right panel) are shown. The values shown are the mean ± SEM. Letters are used to indicate the values that significantly differ from each other ($P < .05$).

Abbreviations: mono, monocytes; Ctrl, macrophage negative control; IL-4, 30 ng/mL IL-4 treatment control; M10, myostatin 10 ng/mL; M50, myostatin 50 ng/mL; M100, myostatin 100 ng/mL.

ER-negative breast cancer. One plausible explanation for the effect of post-diagnosis physical activity may be related to the beneficial effect of exercise on estrogen levels and β-adrenergic signaling.[27,28] Exercise reduced breast cancer development through activation of tumor suppressor Hippo signaling pathway via catecholamine secretion.[28] In addition, Wang et al has shown that exercise-induced epinephrine and interleukin-6 mobilized and redistributed natural killer cells and stimulated apoptosis through Fas/Fas ligand pathway.[29] Several studies have also shown that myokines can reduce tumor proliferation and induce apoptosis.[23,30,31] Hojman et al showed that factors secreted from working muscles inhibited cancer cell growth and induced apoptosis; the addition of anti-myokine antibodies reduced this response by 50%, indicating that myokines may play a role in the protective effect of exercise on cancer.[26,30] In addition, oncostatin M and myostatin inhibited human breast, cervix, and colon cancer cell proliferation and migration, induced apoptosis by mitochondrial metabolic alteration, and decreased tumor cell growth.[23,30,32]

In our study, although myokines increased proportionally according to the intensity of exercise, higher intensity exercise did not result in better tumor response compared to low-intensity exercise. This contrasts with the results of a previous study, which showed a dose-dependent effect of exercise training on tumor attenuation in a mouse model.[33] However, the exercise period in that study was shorter than in our study (3 weeks vs 13 weeks).

Exercise has also been shown to modulate not only tumor growth but also the tumor microenvironment.[31,34,35] Voluntary physical activity has been reported to produce adipose tissue signaling that is unfavorable to tumor growth[31] and to modulate the immune function, thus slowing tumor growth.[34,35] Short-term exercise has been shown to increase the cytotoxic effect of peritoneal macrophages, and cytokines released following exercise are associated with M1 macrophages.[34,35] It has also been suggested that one of the possible mechanisms of exercise on cancer prevention might be regulation of TAM polarization. To test this, we determined whether exercise modulated the TAM population in tumor tissue and found that exercise decreased the number of M2 macrophages but not M1 macrophages.

We showed that steady-low intensity exercise increased M2 macrophage in breast tumor tissues. Polarization of

macrophages could be modulated by extracellular signals, foreign entities or adipokines.[36,37] Previous studies have shown that chronic exercise could affect macrophage polarization in adipose tissues.[38,39] However, its mechanism is still unknown. Since myokines, which were released from working muscle, have been shown to regulate adipocyte and bone differentiation,[40,41] we expected that myokines might regulate macrophage polarization. Among myokines, myostatin has been shown to modulate adipocyte and osteoblastic differentiation and muscle development.[42,43] Therefore, it could be possible that moystatin might regulate the macrophage polarization in tumor tissues.

Previous studies have reported that short- or long-term training could modulate skeletal muscle myostatin expressions in human studies.[24,44-46] Acute training and 6 months of aerobic training reduced muscle and plasma myostatin concentrations,[44,45] however, 10 weeks of high-intensity interval training slightly increased the muscle myostatin expression in obese rheumatoid arthritis and prediabetes patients.[46] Other studies have also shown that myostatin concentration was positively correlated with muscle mass in obese people.[24] Furthermore, cachectic cancer patients, characterized by the loss of skeletal muscle mass, had lower plasma myostatin concentration compared to non-cachectic patents.[21] In this study, only the steady-low intensity group maintained their body weight after tumor inoculation which means that HFLE group may preserve their muscle and fat mass. Even though training could modulate myostatin expression, myostatin is consistently maintained in plasma at rest before and after training and muscle mass might be one of the important factors. Therefore, it is possible that long term exposure of myostatin by steady exercise could affect macrophage polarization during breast tumorigenesis.

In the present study, we found that myostatin inhibited M2 macrophage polarization by suppressing M2 macrophage-associated markers, indicating that myostatin has the potential to be a possible anti-cancer therapy. M2 macrophages are associated with the promotion of tumor progression and metastasis by modulating tumor growth, invasion, cell migration, and angiogenesis.[47,48] Thus, suppressing M2 macrophage polarization is an important therapeutic strategy for inhibiting the development of cancer. Previous studies have found that restricting M2 macrophage polarization suppresses several types of cancer, including breast cancer and colorectal cancer.[49,50] Therefore, it is important to understand the prevalence and role of M1 and M2 macrophages in cancer development. Past research has reported that imatinib, a tyrosine kinase inhibitor, inhibited M2 macrophage polarization by blocking the phosphorylation of STAT6 and nuclear translocation, thus preventing lung cancer metastasis.[51] In the present study, myostatin reduced M2 macrophage polarization by inhibiting the JAK1/STAT6 pathway in macrophage cells differentiated from U937 monocytes. Macrophages that respond to environmental changes, including the presence of various cytokines and growth factors, have been implicated in M1/M2 polarization.[52] M2 macrophages are polarized by the IL-4 or IL-13 activation of JAK1/STAT6, which drives the production of M2 polarization markers, including CD206, Arg1, and Ym1.[52,53] In the present study, myostatin reduced the protein and mRNA expression of M2 polarization markers such as CD163 and Arg1 compared with the IL-4 control. However, this study was limited to in-vitro analysis, so in-vivo research is required to investigate these mechanisms in more detail.

This study has potential limitations. First, only 1 model for development of breast cancer, a high fat diet together with a triple-negative breast cancer model, is used. Breast cancer is actually several different diseases so this study gives just a limited view of exercise and cancer prevention. Second, even though long term exercise has been shown to modulate systemic inflammation, adiposity, and insulin resistance which reduce breast tumor risks, we only focused on macrophage polarization by myostatin. Finally, several myokines are released from the muscle during exercise but we only focused on myostatin. Since other myokines such as interleukin-6, interleukin-15, and irisin showed immunomodulatory responses, their effects also should be investigated.

In conclusion, steady low-intensity exercise before the development of cancer may delay breast cancer growth and reduce tumor volume by decreasing M2 macrophage infiltration into the tumor tissue. Further research should be done on other animal models of breast cancer such as the DMBA model or xenograft that use estrogen-dependent cancer models.

Declaration of Conflicting Interests

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ORCID iD

Jung-Hyun Kim https://orcid.org/0000-0001-5882-4094

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