Effects of Non-motorized Voluntary Running on Experimental and Spontaneous Metastasis in Mice

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Abstract. *The present study investigated the effects of non-motorized voluntary running on experimental metastasis of B16BL/6 melanoma and spontaneous metastasis of Lewis lung carcinoma (LLC) in male C57BL/6 mice. After 9 weeks of running, mice (n=30 per group) received an intravenous injection of B16BL/6 cells or a subcutaneous injection of LLC cells, and then they were continued with their running activities. Experiments were terminated 2 weeks after the intravenous injection of B16BL/6 cells or 2 weeks after surgical removal of the primary tumor from mice subcutaneously injected with LLC cells. Mice in the running group ran an average of 4-6 km/day for the duration of the experiment. Voluntary running reduced body weight compared with the sedentary controls, but there were no differences in the number and size of lung metastases between groups with either model. Voluntary running significantly reduced plasma insulin and leptin levels and increased adiponectin level in mice with and without LLC compared with the sedentary controls. Having LLC significantly increased plasma concentrations of vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), PDGF-AB and monocyte chemotactic protein-1 (MCP-1) in mice. Voluntary running significantly increased plasma PDGF-BB and PDGF-AB, but not VEGF and MCP-1, in mice with LLC compared to their sedentary counterparts. In conclusion, non-motorized voluntary running was favorable to body weight and the expression of related adipokines, but at 4-6 km/day it did not affect either experimental or spontaneous metastasis in mice.*

Epidemiologic studies suggest that physical activity reduces the risk of certain types of cancer, *e.g.* colorectal cancer (1, 2) and breast cancer in humans (3, 4). This risk reduction may be associated with changes in energy expenditure, body composition or dietary practice. Support for the epidemiologic observations includes findings that certain patterns of physical activity reduce experimentally induced tumorigenesis (*e.g.* mammary carcinogenesis (5, 6)) in laboratory rodents. Both epidemiologic and animal studies indicate that physical activity may play a favorable role in primary cancer prevention.

Metastasis, the spread of malignant cells from a primary tumor to different sites of the same organ or to distant organs, is the most devastating aspect of cancer. Its occurrence directly affects the prognosis and survival of cancer patients. Because early diagnosis and treatment of primary tumor have markedly improved the survival rate of cancer patients, there has been great interest in secondary cancer prevention in both general public and research community in recent years. Studies on the relationship between physical activity and metastasis are limited, and data from available laboratory studies (non-motorized voluntary running or forced exercise by treadmill) are inconsistent. While some studies show that voluntary (7) and forced exercise (8) reduce malignant spread, others show no such an inhibitory effect (9), or even that it tends to increase metastatic yield (10). This inconsistency could be a result of variations in the type, duration and intensity of the exercise, the strain of animals used, or the metastatic process investigated.

Physical activity is defined as any bodily movement produced by skeletal muscles that results in energy expenditure beyond resting expenditure, and it is different from exercise that is planned, structured, repetitive, and purposeful in the sense that improvement of maintenance of physical fitness is the objective (11). The purpose of the present study was to investigate the effects of moderate physical activity, achieved by non-motorized voluntary running, on malignant spread in C57BL/6 mice, by using commonly used models of experimental and spontaneous metastasis.

Materials and Methods

This study was approved by the Animal Care and Use Committee of the USDA-ARS Grand Forks Human Nutrition Research Center. The procedures followed the National Institutes of Health guidelines for the care and use of laboratory animals (12).

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Animals and diet. One hundred and twenty three-week-old male C57BL/6 mice (NCI-Animal Production Program, Frederick, MD, USA), average body weight 14.8 g, were housed in a pathogen-free room on a 12:12-hour light-dark cycle and maintained at 22±1˚C, and they were acclimated for one week before the experiment. Mice had free access to pelleted AIN-93G diet (13) and deionized water, and they were weighed weekly. Food intake was recorded daily for 8 consecutive days from the sixth week of the voluntary running.

Cell lines and cell culture. B16BL/6 murine melanoma cells (provided by Dr. Isaiah Fidler, MD Anderson Cancer Center, Houston, TX, USA) were cultured in minimum essential medium (MEM). The medium was supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM MEM-nonessential amino acid solution and 1 mM MEM vitamin solution. Lewis lung carcinoma cells (LLC; provided by Dr. Pnina Brodt, McGill University, Montreal, Quebec, Canada) were cultured with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. Cells from both lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37˚C, and they were periodically monitored for phenotype (microscopically examining the cell morphology), proliferation properties and metastatic capacity by injecting these cells to mice. All assessments of cell identity and behavior were similar to those of original stocks from the institutions providing the cell lines.

Experimental design. We performed two experiments to investigate the effects of non-motorized voluntary running on malignant spread; one was on experimental metastasis, and the other was on spontaneous metastasis. In each experiment, mice were individually housed in wire-topped plastic boxes for the sedentary group (n=30 per group), or in cages with freely accessible in-cage running wheels (Lafayette Instrument, Lafayette, IN, USA) for the physically active group (n=30 per group). Running distance of each mouse was recorded daily for the physically active group. After 9 weeks, experimental metastasis was assessed in mice by an intravenous injection of B16BL/6 cells (0.75×10^5/200 μl/mouse) via the lateral tail vein, and then mice were maintained on their respective treatments for an additional 2 weeks. For spontaneous metastasis, mice were subcutaneously injected with LLC cells (2.5×10^5/50 μl/mouse) into the lower dorsal region. An additional 10 mice in each group were raised without an LLC injection, which were used as controls for plasma cytokine analysis at the end of the experiment. The induced subcutaneous tumor was surgically removed when it reached approximately 1 cm in diameter, and then the mice were maintained on their respective treatments for an additional 2 weeks. At experiment termination, mice were fasted overnight, and then they were injected intraperitoneally with a mixture of ketamine and xylazine; their lungs were harvested and fixed with phosphate-buffered formalin (B16BL/6 melanoma) or phosphate-buffered formalin/Bruin's solution (LLC). The number of pulmonary metastases was counted by using a dissecting microscope, and the cross-sectional area and average diameter of tumors were measured by using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD, USA) and camera-equipped microscope. Tumor volume was estimated with the assumption that tumors were spherical and calculated by using the average diameter measured (14). Abdominal adipose tissues (gonadal and perirenal) were collected and their weights were recorded, soleus muscle from both legs were collected for analysis of citrate synthase activity, and plasma were collected for cytokine quantifications.

Citrate synthase activity. Citrate synthase activity, which mediates the oxidative capacity of skeletal muscle, was used as an index of the physical activity of the mice. Maximal citrate synthase activity was determined spectrophotometrically on soleus muscle homogenates by using the method of Kennedy *et al*. (15). Citrate synthase activity was expressed as μmol/min/mg protein at 25˚C. Protein was quantified according to the Bradford method (Bio-Rad, Hercules, CA, USA).

Plasma cytokine analyses. We quantified plasma insulin and angiogenic cytokines from mice of the spontaneous metastasis experiment. Plasma insulin, leptin, adiponectin, vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), PDGF-AB and monocyte chemotactic protein-1 (MCP-1) were measured by using sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. Samples were read within the linear range of the assay, and the accuracy of the analysis was confirmed by the control cytokine provided in each ELISA kit.

Statistical analyses. Student's *t*-test was performed to compare differences in body weight, citrate synthase activity and pulmonary metastatic yield between sedentary and physically active groups. Two-way ANOVA and Tukey contrasts were used to compare differences in plasma cytokine concentrations between the non-LLC-bearing and LLC-bearing mice, with and without voluntary running. Regression analysis was performed to relate average daily running distance after the injection of cancer cells with metastatic yield, tumor cross-sectional area and tumor volume in mice from the voluntary running group of each experiment. All data are presented as means±SEM. Differences with a *p*-value of 0.05 or less were considered significant. All statistical analyses were performed by using SAS software (version 9.2; SAS Institute, Inc., Cary, NC, USA).

Results

Male C57BL/6 mice ran, on a voluntary basis, approximately 4-6 km/day throughout the experiment (Figure 1), which was similar to the reported daily running distance for this strain (16). The activity of citrate synthase (a catalytic enzyme in the first step of citric acid cycle) of soleus muscle was significantly increased in physically active mice compared to their sedentary controls in both experiments ($p<0.01$; Table I). Voluntary running reduced body weight compared to sedentary mice in both experiments (Figure 2). This difference was statistically significant 2 weeks after the initiation of the running ($p<0.05$), and the significant decrease continued throughout the experiment (except week 4 of the spontaneous metastasis experiment, $p<0.07$). Correspondingly, there was an approximately 30-40% decrease in abdominal adipose weight (gonadal and perirenal) in running mice compared to their sedentary controls in both experiments (data not shown). There was no difference in food intake between groups in the experimental metastasis experiment (3.4±0.1 g/day for both groups; n=10). The intake of the physically active group was slightly, but significantly, higher than that of the sedentary group in the

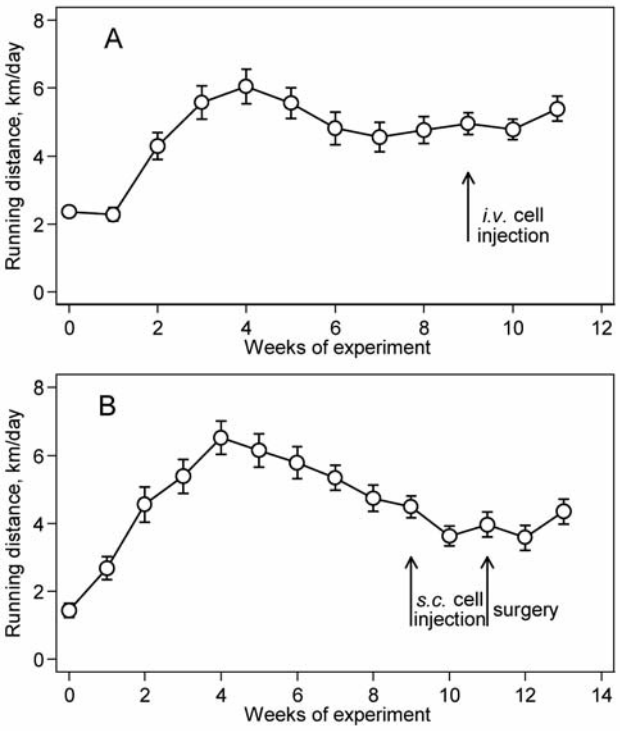

Figure 1. *Average daily running distance of mice determined by using a non-motorized running wheel for the duration of the experiment: (a) experimental metastasis (n=30 per group) and (b) spontaneous metastasis (n=30 per group). Values are mean±SEM.*

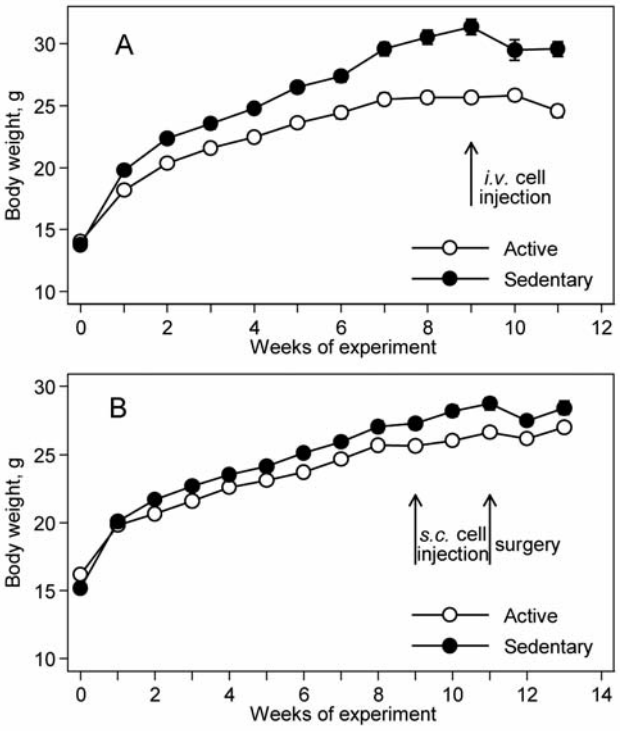

Figure 2. *Body weight changes in mice during the experiment: (a) experimental metastasis (n=30 per group) and (b) spontaneous metastasis (n=30 per group). Student's t-test was used to compare the difference between groups. Voluntary running reduced body weight compared to the sedentary controls. The difference was statistically significant 2 weeks after the initiation of the running ($p<0.05$), and the significant decrease continued throughout the experiment (except week 4 of the spontaneous metastasis experiment, $p<0.07$). Values are mean±SEM.*

spontaneous metastasis experiment (3.5±0.1 g/day *vs.* 3.2±0.1 g/day, $p<0.05$; n=10).

Intravenous injection of B16BL/6 cells and subcutaneous injection of LLC cells resulted in metastatic development and growth in the lungs. There were no significant differences in the number of lung metastases, nor the tumor cross-sectional area and tumor volume between sedentary and running mice with either experimental or spontaneous metastasis model (Table I). Regression analysis showed that the average daily running distance after the injection of cancer cells did not predict tumor numbers, nor tumor cross-sectional area and tumor volume in either experiment (Figure 3); even though there tended to be a slightly inverse relationship between running distance and metastatic tumor yield in the spontaneous metastasis experiment.

Plasma insulin, leptin and adiponectin were quantified in mice from the spontaneous metastasis experiment. Voluntary running reduced plasma insulin (12%; $p<0.05$) and leptin (65%; $p<0.05$) in mice not injected with LLC, and LLC decreased insulin (15%, $p<0.05$) and leptin (45%, $p<0.05$), compared to the sedentary controls (Figure 4A and 4B). Voluntary running did not further reduce plasma insulin (Figure 4A), but significantly reduce plasma leptin in mice with LLC (37%, $p<0.05$; Figure 4B) compared to their sedentary counterparts with LLC. Plasma adiponectin was significantly increased by running ($p<0.01$) and LLC ($p=0.03$); the running did not further increase adiponectin in mice with LLC compared with their sedentary counterparts (Figure 4C).

Plasma angiogenic cytokines VEGF, PDGF-BB, PDGF-AB and MCP-1 were quantified in mice from the spontaneous metastasis experiment. Both voluntary running and LLC significantly increased plasma VEGF ($p<0.01$); the running did not further increase VEGF in mice with LLC compared with their sedentary counterparts (Figure 5A). Voluntary running or LLC did not significantly increase plasma PDGF-BB compared to their sedentary controls; however, the running resulted in a 4-fold increase in PDGF-BB in mice with LLC compared to their sedentary counterparts ($p<0.05$; Figure 5B). Voluntary running did not, but LLC did significantly increase plasma PDGF-AB compared with the non-LLC sedentary controls ($p<0.05$); however, the running increased PDGF-AB in mice with LLC

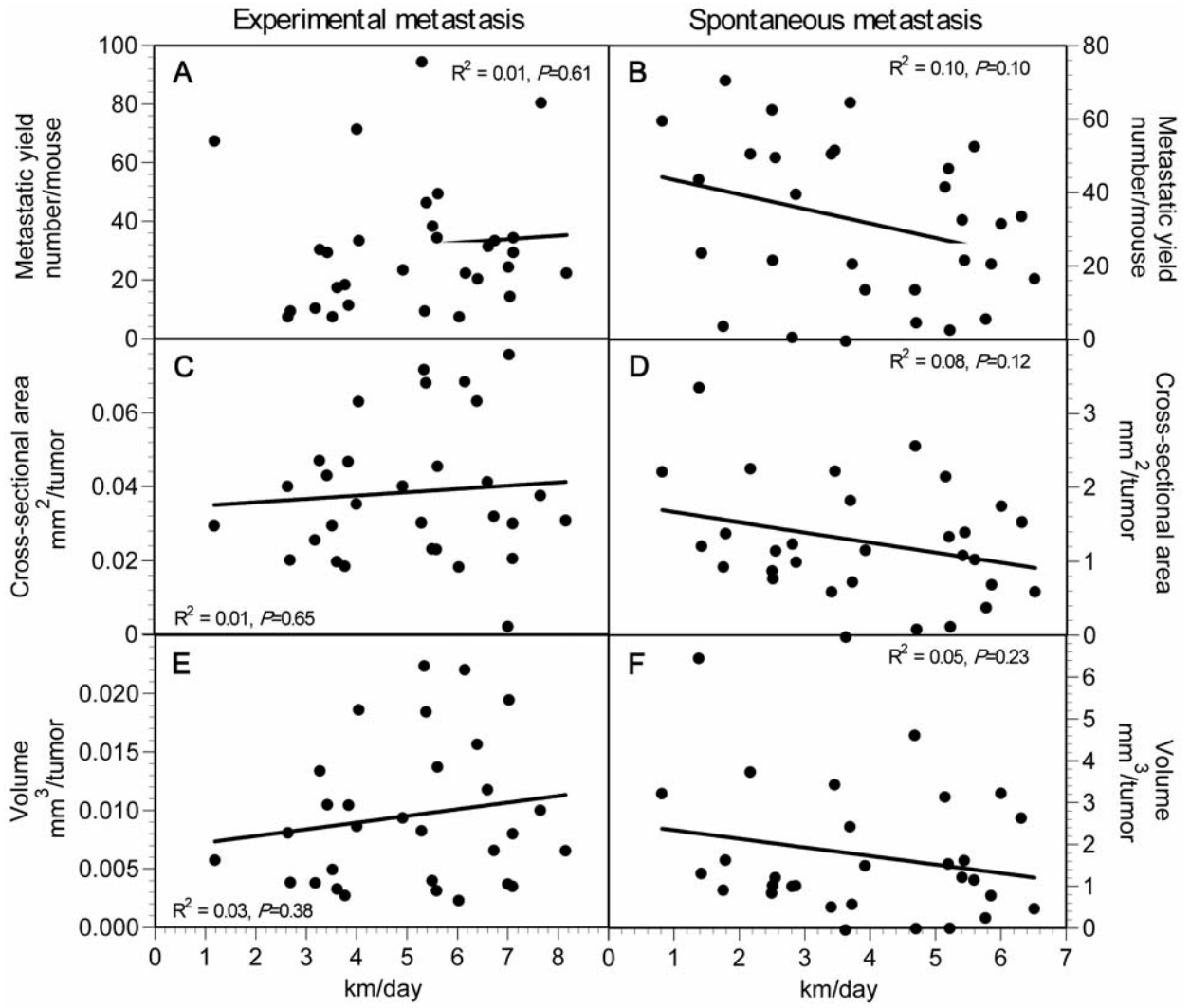

Figure 3. *Regression analysis of average daily running distance after the injection of cancer cells vs. metastatic tumor yield (a, b), tumor cross-sectional area (c, d) and tumor volume (e, f) in mice intravenously injected with B16BL/6 melanoma cells (left column) or subcutaneously injected with LLC cells (right column).*

Table I. *Effects of voluntary running on citrate synthase activity and pulmonary metastatic yield in male C57BL/6 mice*[a].

	Sedentary (n)	Active (n)	*P*-value
Experimental metastasis[b]			
Citrate synthase, μmol/min/mg protein	1.01±0.04 (29)	1.25±0.04 (30)	<0.01
Metastatic tumor yield, number/mouse	34±4 (28)	32±4 (30)	0.71
Tumor cross-sectional area, mm^2	0.044±0.003 (28)	0.039±0.002 (30)	0.23
Tumor volume, mm^3	0.012±0.001 (28)	0.010±0.001 (30)	0.17
Spontaneous metastasis[c]			
Citrate synthase, μmol/min/mg protein	1.57±0.03 (27)	1.98±0.06 (29)	<0.01
Metastatic tumor yield, number/mouse	32±3 (30)	32±4 (30)	0.96
Tumor cross-sectional area, mm^2	1.384±0.064 (30)	1.517±0.071 (30)	0.44
Tumor volume, mm^3	1.928±0.138 (30)	2.136±0.171 (30)	0.48

[a]Values are mean±SEM. [b]Experimental metastasis was assessed by using B16BL/6 melanoma cells with an intravenous injection model. [c]Spontaneous metastasis was assessed by using Lewis lung carcinoma cells with a subcutaneous injection model.

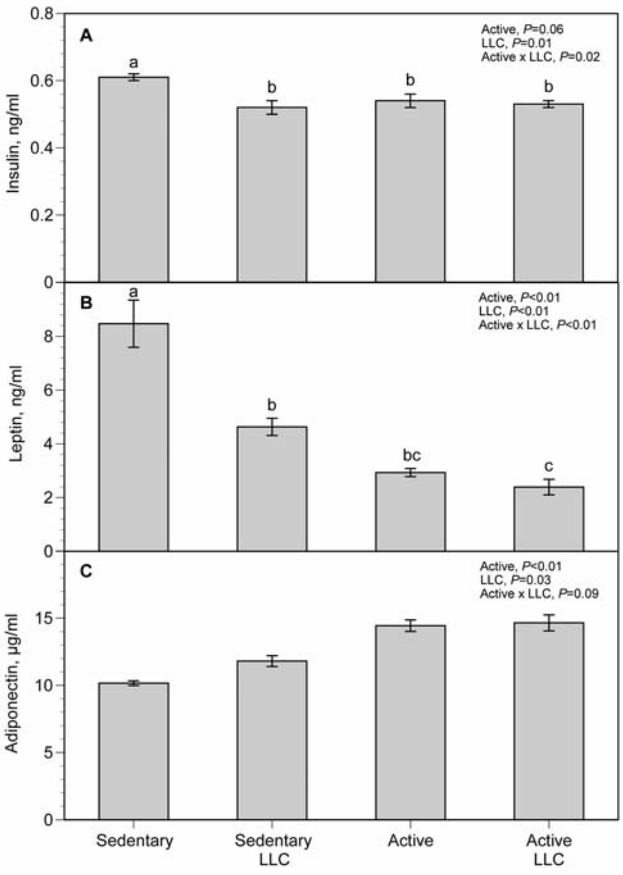

Figure 4. *Plasma concentrations of insulin (a), leptin (b) and adiponectin (c) in sedentary and physically active mice with and without a subcutaneous injection of LLC cells. Two-way ANOVA was used to determine two main effects (voluntary running and LLC) and their interaction, and Tukey contrasts were performed if the interaction was statistically significant ($p \leq 0.05$). Values with different letters are statistically significant at $p \leq 0.05$ by Tukey contrasts (n=10 for each data point). Values are mean±SEM.*

by 85% compared to their sedentary counterparts ($p<0.05$; Figure 5C). There was no difference in plasma MCP-1 between sedentary and physically active groups ($p=0.80$, Figure 5D). Lewis lung carcinoma significantly increased MCP-1 in both sedentary and running groups compared to their respective non-LLC counterparts ($p<0.01$, Figure 5D).

Discussion

The present study showed that non-motorized voluntary running did not affect the number and size of lung metastases. We used two animal models in this study. The experimental metastasis model assessed malignant spread from extravasation to the formation of secondary tumors in the lungs, and the spontaneous metastasis model assessed entire process of metastasis from formation of a primary tumor to the development of metastases in the lungs. Our results indicate that voluntary running at an average of 4-6 km/day does not affect the metastatic process as assessed with either model in male C57BL/6 mice.

Voluntary running on a non-motorized wheel and forced running by treadmill are two commonly used models of physical activity in laboratory rodents. We used the voluntary running because the mice themselves determined the frequency, duration and intensity of the exercise and ran in a self-controlled, physically capable manner. This is an advantage over forced running because reinforcement-associated stress is a potential confounder that may alter the treatment effect. For example, swim-induced stress resulted in a two-fold increase in lung metastases in rats (17). The limitation of voluntary running is variation in daily activity (*e.g.* variation in daily running distance), which may affect the outcome of the study.

The aggressiveness of cancer cells and the experimental procedures may affect the effect of physical activity on malignant spread. Lewis lung carcinoma cell line is a moderately aggressive cell line (18), and it has the capability of rapid spread from a primary tumor to target organs. Intravenous injection of B16BL/6 cells eliminates the step of intravasation, which simplifies the metastatic cascade, and allows cells have a relatively easy access to their target organ. An early study showed that treadmill running reduces the lung retention of cells from a less aggressive cell line after an intravenous injection compared to cells from a more aggressive line (9).

The actions of insulin, leptin and adiponectin are closely interrelated. Insulin secretion occurs in direct response to food intake, and leptin regulates body weight through its effects on food intake and energy expenditure (19). Adiponectin modulates glucose regulation and fatty acid catabolism (20), and a higher level of plasma adiponectin is associated with greater insulin-stimulated glucose utilization (21). In the present study, voluntary running reduced plasma concentrations of insulin and leptin and increased plasma adiponectin compared with the sedentary controls, and these changes were consistent with changes in body weight and abdominal adiposity between groups. These results indicate that voluntary running affect energy expenditure and adipogenesis in a favorable manner; however, such favorableness does not affect spontaneous LLC metastasis.

Decreases in plasma insulin and leptin and an increase in adiponectin in sedentary mice with LLC compared to their non-LLC counterparts suggest that energy expenditure is increased with metastasis. This suggestion seems to be reasonable because although both normal and cancer cells use glucose and glutamine as substrates to generate energy, rapidly dividing cells require increased energy for their metabolism and proliferation. Many cancer cells satisfy their energy need by taking up larger amounts of glucose than do

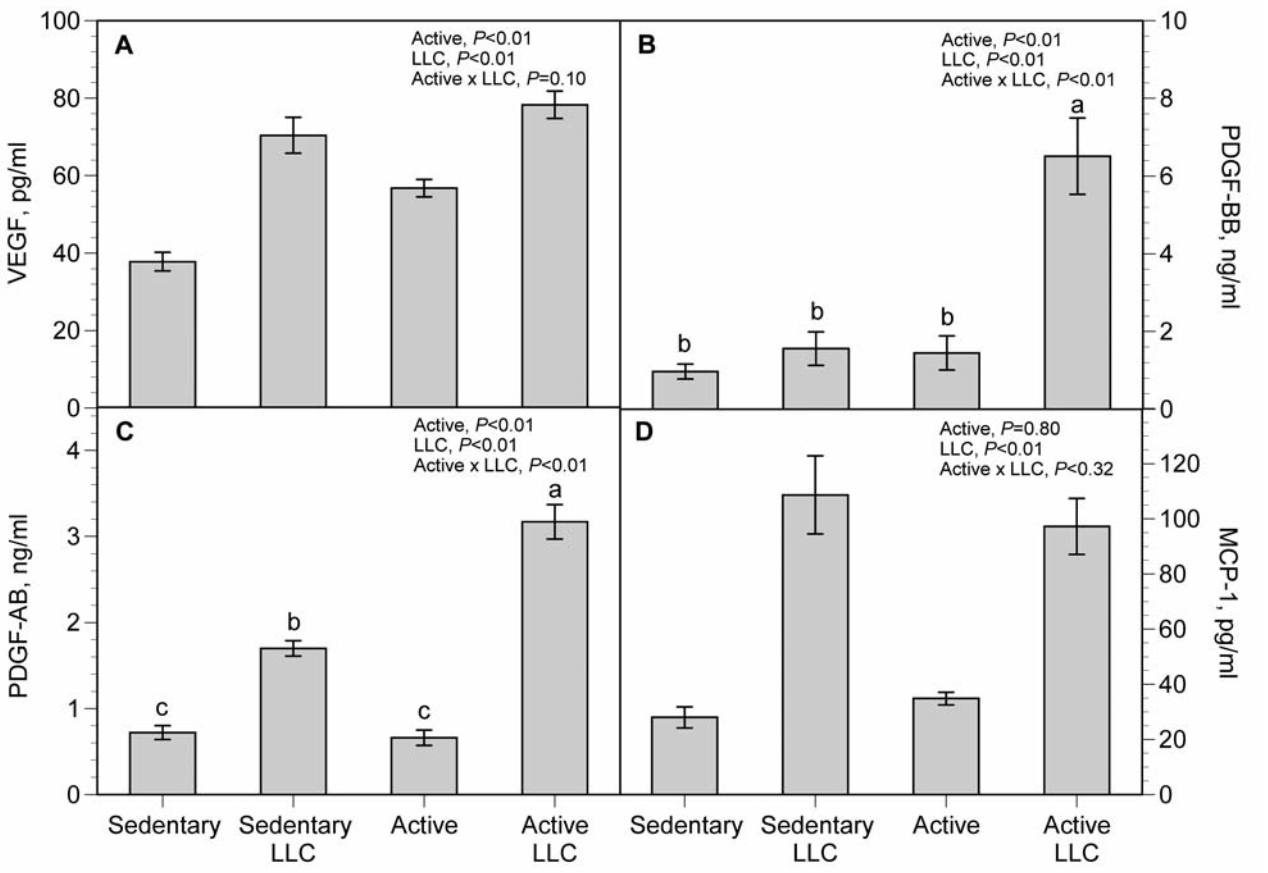

Figure 5. *Plasma concentrations of vascular endothelial growth factor (VEGF) (a), platelet-derived growth factor-BB (PDGF-BB)(b), PDGF-AB (c) and monocyte chemotactic protein-1 (MCP-1)(d) in sedentary and physically active mice with and without a subcutaneous injection of LLC cells. Two-way ANOVA was used to determine two main effects (voluntary running and LLC) and their interaction, and Tukey contrasts were performed if the interaction was statistically significant (p≤0.05). Values with different letters are statistically significant at p≤0.05 by Tukey contrasts (n=10 for each data point). Values are mean±SEM.*

normal cells. Metabolic patterns change, however, over time in tumor-bearing animals, with an initial hyper-metabolic phase followed by a decrease to a pre-terminal hypo-metabolic phase (22). In humans, cancer in a specific site (*e.g.* lung cancer) is particularly associated with elevated resting energy expenditure (23, 24).

Angiogenesis, a complex process that involves participation of multiple cytokines, plays a critical role in metastasis. Vascular endothelial growth factor and PDGF have potent angiogenic activities and are principal cytokines in tumor angiogenesis. Overexpression of VEGF (25, 26) and PDGF (27, 28) are associated with advanced tumor stages and unfavorable prognosis in cancer patients. Furthermore, VEGF promotes angiogenesis by triggering signaling pathways, including MCP-1 (29), which in turn up-regulates VEGF production (29). High serum MCP-1 is associated with lymph node metastasis in breast cancer patients (30). Results from the present study are consistent with these observations. The significant increases in plasma VEGF, PDGFs and MCP-1 in mice with LLC compared with non-LLC counterparts suggest enhanced angiogenesis during the metastatic development and growth in the lungs.

Increases in blood angiogenic cytokine concentrations in tumor-bearing mice may be related to hypoxic stimulation of angiogenesis by tumors. The distance between tumors, or cells in tumors, and adjacent blood vessels (*e.g.* ≥100 μm, the diffusion limit for oxygen) and the irregular pattern and organization of tumor vasculature create hypoxia, which increases production and secretion of multiple angiogenic cytokines by tumors (31), and these cytokines, including VEGF and PDGF, are synergistic in neovascularization (32, 33). During physical exercise, more blood goes to major organs through normal vasculature to maintain physiological oxygen needs; this may further deprive tumors of oxygen and increase their production of angiogenic factors. In the present study, voluntary running significantly increased plasma PDGF-BB and PDGF-AB, but not VEGF and MCP-1, in mice with LLC, suggesting that the physical activity-mediated production of

these cytokines in tumor-bearing mice was through different mechanisms. Interestingly, the significant increase in PDGFs in running mice with LLC was not correlated with an increase in pulmonary metastatic yield. Thus, further investigations are warranted on physical exercise and PDGF production and its relation to angiogenesis and malignancy.

Voluntary running at 4-5 km/day did not affect metastasis with the models tested in the present study. However, this does not indicate that moderate physical activity may not play a role in secondary cancer prevention. Obesity, after tobacco use, is the single greatest risk factor for cancer. Increased recurrence and metastasis have been reported with obese cancer survivors (34, 35) and diet-induced obese rodents (36, 37). Physical activity increases energy expenditure, reduces body adiposity and changes obesity-related dysregulation of inflammatory cytokines and hormones. Thus, physical activity may be a useful adjuvant in reducing the risk of obesity-enhanced secondary cancer development and growth; this remains to be investigated.

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Conflict of Interest

The Authors declare no conflict of interest.

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